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DATA DEVELOPMENT STRATEGY FOR EVALUATION OF OCCUPATIONAL HEALTH HAZARDS OF NEW CHEMICALS OF INTEREST TO THE AIR FORCE

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

//SIGNED//

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New chemicals developed for the use by the Air Force must undergo evaluation to determine if these chemicals will be detrimental to the health of Air Force personnel. Chemical safety assessment is a costly and time consuming process. This report is a description of recommended procedures for toxicity testing of new chemicals. A strategy for the progressive and logical development of toxicology data that are needed for health hazard characterization, and ultimately for risk assessment is presented. The overall cost and time requirements of the proposed testing strategy are provided. The relationship between the amount of toxicity testing conducted and the level of confidence in the toxicity classification of new chemical is emphasized.

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PREFACE

This report is one of a series of reports describing the research efforts of the Predictive Toxicology program at AFRL/HEST. The objectives of this task is to develop an overall perspective of the toxicity testing requirements for health safety evaluation of new chemicals developed for use by the Air Force. The research described in this report began in June 1999 and was completed in March 2000. This work was financially supported by the Air Force Office of Scientific Research (2312A202). Technical support was provided by ManTech Geo-Centers Joint Venture F41624-96-C-9010. Maj Steven Channel served as Contract Technical Monitor for AFRL/HEST. No animals were used in the studies described in this document.

A significant portion of this report was provided to the Air Force by Toxicology/Regulatory Services (TRS), Charlottesville, VA under a subcontract to ManTech Geo-Centers Joint Venture. The scope of work for the subcontract stated:

> "Expert opinion and advice are requested for an informational document to describe acceptable strategies for occupational safety evaluation of new chemicals of interest to the Air Force. Chemicals are those that originate from the Air Force R&D laboratories and more closely resemble industrial chemicals than drugs or pesticides. Chemical warfare agents are not included. Emphasis should be placed on discussing the value added for including specific testing protocols in the overall testing strategy. The questions being asked are: Given the data provided by this testing strategy, how confident would decision makers be that the health of Air Force personnel and contractors was adequately protected? Would any additional tests, not included in the basic test strategy proposed, merit consideration for a second testing tier that would provide additional confidence in the decision making process? Estimates of cost and time for performance of the proposed testing strategy are to be included in the document. Discussion should be limited to toxicity testing that best supports human health considerations."

The document provided by TRS was revised, resulting in this report.

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FIGURES AND TABLES

ABBREVIATIONS

ADI	Acceptable Daily Dose
AFRL	Air Force Research Laboratory
AG	8-azaguanine
CAS RN	Chemical Abstracts Service registry number
CCRIS	Chemical Carcinogenesis Research Information System
CPSC	Consumer Product Safety Commission
DART	Developmental and Reproductive Toxicology
DNA	Deoxyribonucleic Acid
DOT	Department of Transportation (DOT)
DSD	Dangerous Substances Directive
EDI	Estimated Daily Intake
EHD	Estimated Human Dose
EMIC	Environmental Mutagen Information Center
EMICBACK	Environmental Mutagen Information Center Backfile
EPA	Environmental Protection Agency
ETICBACK	Environmental Teratology Information Center Backfile
EU	European Union
GPMT	Guinea-pig maximization test
HEST	Operational Toxicology Branch
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HSDB	Hazardous Substances Data Bank
IUCLID	International Uniform Chemical Information Database

LC_{50}	Lethal Concentration for 50% of exposed animals
LD_{50}	Lethal Dose for 50% of exposed animals
LOAEL	lowest-observable-adverse-effects-level
mg/kg	milligrams per kilogram
mg/l	milligrams per liter
NCP	New Chemicals Program
NOAEL	no-observable-adverse-effects-level
OECD	Organization for Economic Cooperation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
OSHA	Occupational Safety and Health Administration
PMN	pre-manufacturing notification
QSAR	Quantitative Structure-activity Relationship
RTECS	Registry of Toxic Effects of Chemical Substances
SAR	Structure-activity Relationship
STD	Standard Temperature and Pressure
TFT	Trifluorothymidine
TG	6-thioguanine
TK	Thymidine Kinase
TRS	Toxicology/Regulatory Services
TSCA	Toxic Substances Control Act
TSCATS	Toxic Substance Control Act Test Submission
VSD	Virtually Safe Dose
WHMIS	Workplace Hazardous Materials Information System

ABSTRACT

New chemicals developed for use by the Air Force must undergo evaluation to determine if these chemicals will be detrimental to the health of Air Force personnel. Chemical safety assessment is a costly and time consuming process. This report is a description of recommended procedures for toxicity testing of new chemicals. A strategy for the progressive and logical development of toxicology data that are needed for health hazard characterization, and ultimately for risk assessment is presented. The overall costs and time requirements of the proposed testing strategy are provided. The relationship between the amount of toxicity testing conducted and the level of confidence in the toxicity classification of a new chemical is emphasized.

EXECUTIVE SUMMARY

The strategy presented in the current document employs the best features of current U.S. and European Union (EU) new chemical regulatory schemes, as well as common chemical industry practices, first by evaluating thoroughly all relevant existing information and then by conducting a strategic data development program to provide sufficient information to complete the database necessary for a comprehensive occupational health hazard evaluation.

Inherently, there is uncertainty in health hazard characterization. Uncertainty can be reduced through strategic data development programs. The specific data requirements for hazard characterization data for a given chemical cannot be predicted *a priori*, since each chemical has its own unique characteristics. A four tier strategy for the progressive and logical development of toxicology data that are useful for health hazard characterization, and ultimately for risk assessment, is presented in this document.

The lowest tier, Level 1, requires no animal studies. It serves to collect all of the available data that can be generated from theoretical evaluations and *in vitro* studies, as well as data that can be derived from the literature on related chemicals. The second tier of the data development strategy, Level 2, involves the standard short-term tests for toxicity, including acute toxicity, eye and skin irritation and other evaluations. The third tier, Level 3, extends the database to subchronic toxicity endpoints and investigates the kinetics and metabolism of the chemical in test animals. Level 4 studies are long term animal studies for chronic toxicity, carcinogenicity and reproductive toxicity. At each level of data development, several specific testing activities are proposed. It is not intended that all tests be performed at each level.

Hazard-based assignments of levels of toxicological concern are used early in the development or use of a new chemical. However, when adequate toxicology data become available, it is preferred to use risk assessment rather than hazard-based assignment for decision-making related to chemical safety and/or the need for personal protection from occupational chemical exposures. Risk assessment is an iterative process whereby the application of new toxicology and/or exposure data may be used to improve the quality and accuracy of the risk characterization.

Specific recommendations for the conduct of a systematic toxicological evaluation of new chemicals are presented.

INTRODUCTION

This report focuses on detailed practical guidance for characterization and evaluation of the occupational health hazards of new chemicals developed by the Air Force. For the purposes of this report, it is assumed that these chemicals have never been used commercially and, therefore, there is no existing toxicology and/or exposure data available at the beginning of this process. In this case, the process of "health hazard characterization" is used to identify the most biologically significant effects elicited by these chemicals and to determine the no-observed-adverse-effect level (NOAEL) for those effects, so that safe occupational exposure levels may be defined.

The strategy presented in the current document exploits the best features of current new chemical regulatory schemes, as well as common chemical industry practices, first by evaluating thoroughly all relevant existing information and then by conducting a strategic data development program to provide sufficient information to complete the database necessary for a comprehensive occupational health hazard evaluation. A multilevel data development strategy is outlined below, beginning with an evaluation of existing information, developing a basic toxicological data set and then, depending on the advice of an expert panel of scientists, further development of a more comprehensive toxicology database, as appropriate.

Multidimensional Perspective of Toxicological Evaluations

Arriving at a definitive statement concerning the toxicity of a chemical is a difficult task. A statement that "a chemical is toxic", "a chemical is an irritant", "a chemical is a carcinogen" is not adequate. All chemicals will exhibit toxic properties at some dose. Therefore, it is not a question of whether a chemical is toxic, but rather at what dose will it become toxic. The dose-response relationship becomes the key issue relating to the safe deployment of chemicals in the operational environment.

There are several important factors that must be taken into consideration before making any statements regarding the toxicity of a chemical. As a generalization, it may be concluded that our knowledge of toxicity depends on how we look for it. First, there are many types of exposure patterns. A chemical may cause little effect at a particular dose in an acute scenario, i.e., immediate effects following a single exposure, but the same dose may cause severe toxicity if repeated over time. The implication is that toxicity studies conducted over short periods of time (single exposure followed by observations over a limited time) are not adequate to protect health if repeated exposures over long periods of time are expected. Second, there are many potential forms of toxicity, such as acute lethality, developmental teratogenicity and cancer, or those producing permanent or progressive pathology. Usually it is required to conduct specific experimental studies to evaluate different forms of toxicity. Information about the carcinogenicity of a chemical cannot be derived from acute toxicity studies. If such specialized studies are not conducted, then potential health hazards may be overlooked that will only become evident when the chemical is in use and exposed personnel develop adverse health conditions. Third, there are many targets for toxicity - liver, kidney, brain, etc. Toxicity testing must be designed to identify the potential targets so that if a chemical is deployed in a weapons system, health effects in operational personnel can be monitored in an effective manner. Finally, our level of understanding

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of toxicity depends on the nature of experimental tools we use to evaluate effects. Table 1 is a list of representative measurements used in *in vivo* toxicity studies to characterize adverse effects. In general, the standard measurements taken in these studies provide little information on the mechanisms of toxicity, i.e., how the chemical produces its effects. If information about the mechanism of action is desired then additional studies are conducted, often using *in vitro* methods, to elucidate the molecular events involved. Mechanistic knowledge of how a chemical produces its effects is valuable when trying to extrapolate toxicity information obtained from animal studies to man. The strategy to develop toxicological data for new chemicals described below is designed to take these issues into consideration.

General
Body Weight
Food and Water Intake
Behavior
Pathology
Gross Examination of Tissues
Tissue Weights
Histological Examination of Tissue Slices
Differential Blood Cell Counts
Pulmonary Lavage
Clinical Observations
Body Temperature
Heart Rate
Blood Pressure
Sperm Count
Pulmonary Function
Clinical Chemistry
Hematocrit
Blood Urea Nitrogen (BUN)
Hormone Levels
Proteinurea
Enzyme Biomarkers

Table 1: Toxicological Parameters Evaluated in In Vivo Toxicity Studies

Relationship Between Available Information and Confidence in Decisions

The more toxicological information you have the better the chances of identifying toxicological hazards and ultimately making the correct decision for hazard management. The following hypothetical scenario, illustrated in Figure 1, demonstrates the value added by each successive toxicity test when conducting a strategic data development program. Initially, when there are no experimental data available for a new chemical, the uncertainty regarding the nature of potential health effects and the dose level at which they might appear is very difficult, if not impossible, to predict. Quantitative structure-activity relationships (QSAR) and/or toxicological data for related chemicals may be of some use to suggest potential toxicity problems, but provide little reliable

data for estimating the NOAEL. Let us assume that the first experimental study performed, a fixed dose acute toxicity test (Toxicity Data Set 1 in Figure 1), had a measured lowest-observable-adverse-effects-level (LOAEL) of 2000 mg/kg for the endpoint of lethality (i.e., mortality was observed only at the 2000 mg/kg dose level). At the next lower dose level (500
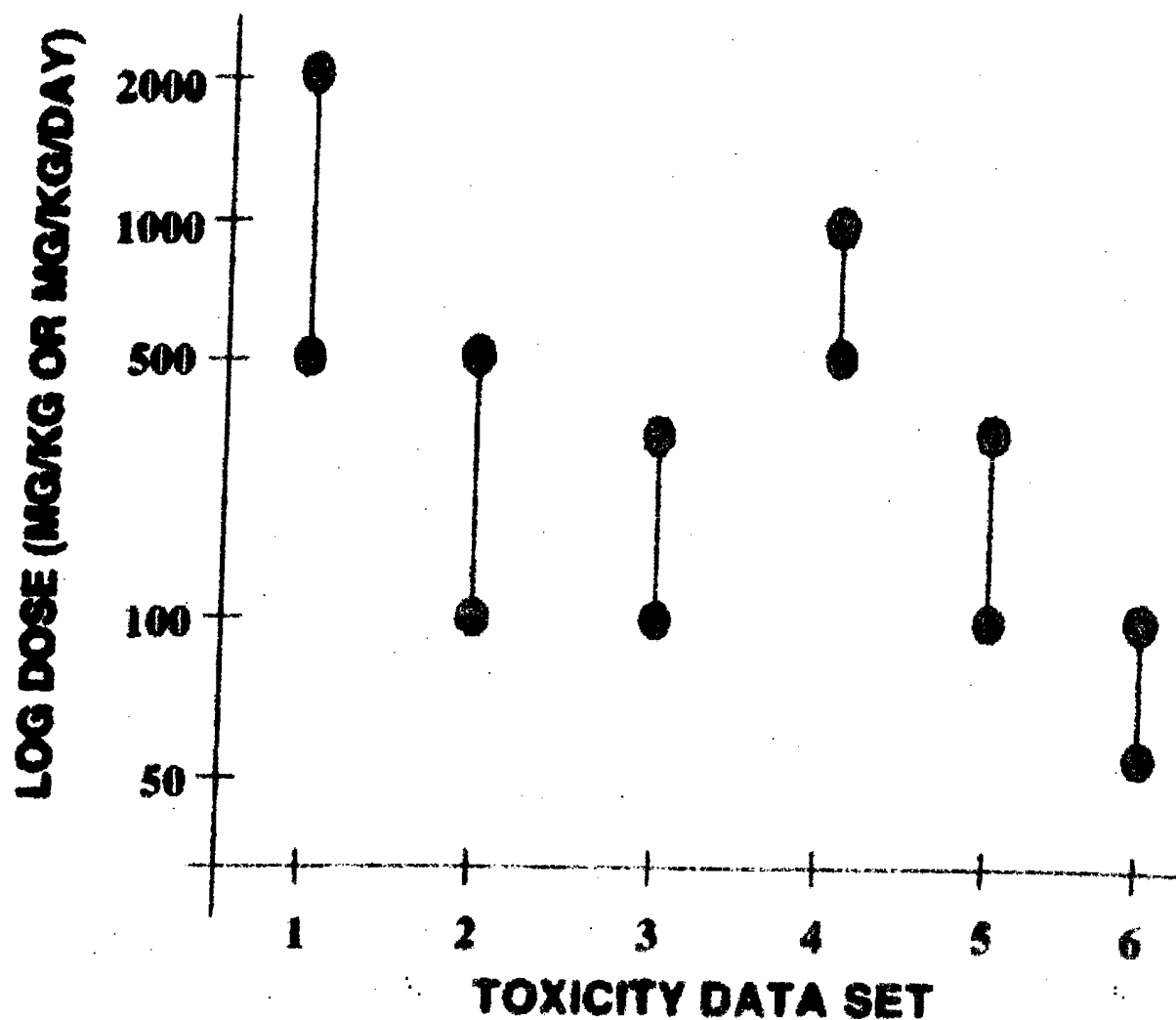
Figure 1: Representative data from a progressive toxicity testing paradigm with increasing complexity and cost to a new chemical. The data shows the relationship between additional toxicity data and estimation of NOAEL.*. For each data set, the top symbol indicates the lowest observable adverse effect level (LOAEL) and the bottom symbol indicates the no observable adverse effect level (NOAEL).

* This is a fictitious testing scenario as described in the text. The various toxicity data sets refer to the results of a series of toxicity tests. The first test (Toxicity Data Set 1) is an acute toxicity test, Data Set 2 is for a 28 day repeated dose study, Data Set 3 is for a 90 day sub-chronic study, Data Set 4 is for a developmental toxicity study and Data Set 5 is for a 2 generation reproductive toxicity study and Data Set 6 is for a 2-year chronic toxicity/oncogenicity bioassay)

mg/kg) no toxicity was observed, thus the observed NOAEL would be 500 mg/kg as indicated in the figure. The actual NOAEL for acute lethality lies somewhere between 500 and 2000 mg/kg. The precise identification of the NOAEL is limited by the experimental design. Based on this single study, useful information about acute toxic effects of the chemical is obtained. However, this information is not adequate to give confidence in situations where repeated exposure to the chemical can occur over time. The second study performed (Toxicity Data Set 2) was a 28-day repeated dose toxicity study at several doses. The doses selected for this study were based on the data obtained in the acute toxicity study. The data obtained indicate that the LOAEL is 500 mg/kg/day for an increase in liver weight, the most sensitive toxicity endpoint identified in this study. The observed NOAEL for this endpoint was 100 mg/kg/day. In this case, repeated dosing results in a non-lethal pathology that has implications for potential carcinogenicity of the chemical. As a consequence of the concerns raised by this study, a third study, a 90-day subchronic toxicity study (Toxicity Data Set 3), was conducted. The results of the histopathological evaluations of tissues indicate that hepatocyte hypertrophy and proliferation could be demonstrated at a LOAEL of 300 mg/kg/day. A NOAEL of 100 mg/kg was obtained for all observed parameters. Due to the concern that women of child baring age may come into contact with the chemical, a fourth study, a developmental toxicity study (Toxicity Data Set 4), was conducted. Rib anomalies were observed at the highest dose level (1000 mg/kg/day) and a NOAEL of 500 mg/kg/day was identified. Although the NOAEL for the developmental study was higher than that for the 90 day subchronic study, thus indicating a lower sensitivity for the developmental effects, the observation of developmental effects at high doses raises concerns about exposure of pregnant women as a special population. The fifth study, a two-generation reproduction study (Toxicity Data Set 5), had reduced body weight gains in offspring of the mid- and high-dose groups with a LOAEL of 300 mg/kg/day and a NOAEL of 100 mg/kg/day. The sixth study, a two-year chronic toxicity/oncogenicity bioassay (Toxicity Data Set 6), had a LOAEL of 100 mg/kg/day based on a dose-related incidence of liver tumors at the higher test doses. No adverse health effects were observed for any toxicity parameters at the 50 mg/kg/day dose level.

This hypothetical testing scenario illustrates two important points. First, as additional testing is conducted, a wider range of possible forms of toxicity are explored, thus allowing for the identification of the most sensitive outcome, in this case cancer, with a reasonable level of confidence. Secondly, by accumulating this extensive database for the toxicity of the chemical, greater confidence is developed that the safe level of exposure can be accurately established. If testing of the chemical had stopped after the second test, then the NOAEL would have been 100 mg/kg/day. Using the default uncertainty factors to build in additional safety when extrapolating from animal data to human beings, the rat NOAEL would be divided by a factor of 10 for uncertainties in extrapolating from animals to man, another factor of 10 to protect the most sensitive human beings, and a third factor of 10 due to the limited toxicity data available. Thus, the safe exposure level (referred to as the reference dose) for this chemical would be 0.1 mg/kg/day. However, if the entire toxicity data set was developed as described above, it would be determined that an exposure of 50 mg/kg/day should not result in any adverse health effects in the rat with a high level of confidence. Now, this NOAEL is lower by a factor of 2 than that identified when only two tests were conducted, however when the animal-to-man extrapolation is conducted the default uncertainty factors are reduced due to the availability of a large toxicity database, i.e., only a factor of 10 for species extrapolation and a factor of 10 for the most

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sensitive individuals would be used. Thus, the calculated reference dose for human exposure would be 0.5 mg/kg/day, and this level would be considered safe for human exposure with a relatively high level of confidence. Comparing the reference doses derived under the two scenarios indicates that the additional toxicity testing will not only allow for a higher reference dose, by a factor of 5, but the level of confidence is significantly greater when all major toxicity issues have been identified. It should be kept in mind that small changes in the levels to which exposure to chemical is regulated can result in significant lifetime costs for protection and remediation.

The drawback to the extended testing approach described above is that it is very expensive to conduct all of the testing and would take several years to generate the complete database. In cases where there is reasonable expectation that large numbers of people will be exposed to the chemical, there is no choice but to conduct an extensive toxicity evaluation before the chemical is used. In other cases where exposure is limited to a select (usually controlled) population and the overall level of production of the chemical is relatively small, a reduced toxicity database may be used to make risk management decisions, but it must be recognized that the level of confidence in the safety evaluation is reduced under these circumstances.

Regulatory Approaches to the Evaluation of New Chemicals

The U.S. Toxic Substances Control Act (TSCA) (1976) regulates all industrial chemicals manufactured, processed or imported into the U.S., except for chemicals already regulated under other laws. Similarly, the European Union (EU) Dangerous Substances Directive (1967) covers all industrial chemicals marketed, processed or imported into the EU, except for chemicals already regulated under other laws.

Under the TSCA pre-manufacturing notification (PMN) scheme, if the Environmental Protection Agency (EPA) suspects that a chemical may pose an unreasonable risk, but lacks sufficient data to take action, TSCA allows EPA to require testing to develop the necessary data. Also, EPA may order testing if the new chemical will be produced in substantial quantities that may result in significant human exposure, the effects of which can not be predicted based on existing information. To enable evaluation of new chemicals before humans are exposed, manufacturers must notify the EPA at least 90 days prior to manufacturing of the new chemical. Although the PMN submission must include any health effects data the manufacturer possesses, EPA is not empowered to require that manufacturers routinely conduct testing of all new chemicals to allow an evaluation of their risks. In fact, a large proportion of PMNs are submitted without any health effects data on the new chemical.

In contrast to the TSCA PMN approach, the EU premarketing notification requires that companies that intend to market new chemicals at greater than one metric ton per year must develop a "base set" of data on the substance and submit a technical dossier at least 60 days prior to marketing the substance in the EU. The health effects testing requirements are acute toxicity tests by two routes of administration, skin and eye irritation tests, a skin sensitization test, two mutagenicity studies, a 28-day repeated dose toxicity study by the most appropriate route of

exposure, and toxicokinetic and preliminary risk assessments. Additional data development is required as the chemical is marketed at higher tonnage triggers.

The respective chemical notification schemes in the U.S. and the EU approach the evaluation of new chemicals very differently. Whereas EPA is authorized to order health effects testing only in limited situations, the EU scheme requires notifying companies to develop and submit a base set of data for most new chemicals. On the other hand, EPA is permitted to intervene at a much earlier stage of the new chemical development process by requiring notification prior to manufacturing, rather than prior to marketing, as is the case in the EU. The intention of the strategy presented in this document is to exploit the best features of both of these chemical regulatory schemes by first evaluating thoroughly all relevant existing information and then by conducting a strategic data development program to provide sufficient information to complete the database necessary for a comprehensive occupational health hazard evaluation.

Overview and Rationale for Proposed Data Development Plan

Information for hazard characterization and risk assessment should be developed logically and sequentially, beginning with basic information and then moving on to more detailed and complex information. Iterative evaluation of data is necessary to guide the data development process for health hazard characterization. Inherently, there is uncertainty in health hazard characterization. Uncertainty can be reduced through a strategic data development program, such as the one described in this document, that improves the toxicology database for a new chemical.
The specific data requirements for hazard characterization data for a given chemical cannot be predicted *a priori*; each chemical has its own unique characteristics. The ultimate size of the database should be proportional to the degree of toxicity seen and anticipated exposure. A four level strategy for the progressive and logical development of toxicology data that are useful for health hazard characterization, and ultimately for risk assessment, is presented below.

Generally, a complete risk assessment is not feasible for new chemicals because experimental exposure data are not available and estimation of exposure may be surrounded by a great degree of uncertainty. Therefore, it may be appropriate under these circumstances to use only hazard assessment data for decision-making related to chemical development and/or requirements for personal protection from occupational chemical exposures. Hazard assessment is based on the experimental results obtained from a variety of toxicological studies, many of which are defined by standard testing guidelines provided by the EPA (OPPTS Series 870) and the Organization for Economic Cooperation and Development (OECD, see Table 2).

Toxicology Study Type	Test Guidelines	Value Added for Study Type
Acute toxicity battery		
• Acute oral toxicity	OPPTS 870.1100 OECD 401, 420, 423 and 425	Evaluation of acute toxicity by oral route; useful for identifying target organs.
• Acute dermal toxicity	OPPTS 870.1200 OECD 402	Evaluation of acute toxicity by dermal route; useful for estimating dermal absorption relative to oral route and identifying target organs.
• Acute inhalation toxicity	OPPTS 870.1300 OECD 403	Evaluation of acute toxicity by respiratory route; useful for identifying target organs.
• Skin irritation	OPPTS 870.2500 OECD 404	Evaluation of potential skin irritation.
• Eye irritation	OPPTS 870.2400 OECD 405	Evaluation of potential eye irritation.
• Skin sensitization	OPPTS 870.2600 OECD 406	Evaluation of potential for skin sensitization.
Genetic toxicity battery		
• *In vitro* bacterial gene mutation	OPPTS 870.5100 OECD 471 and 472	Initial evaluation of mutagenic potential.
• *In vitro* mammalian cell cytogenetics	OPPTS 870.5375 OECD 473	Initial evaluation of mutagenic potential.
• *In vitro* mammalian cell gene mutation	OPPTS 870.5300 OECD 476	Alternate test for initial evaluation of mutagenic potential.
• *In vivo* mammalian micronucleus	OPPTS 870.5395 OECD 474	Second tier evaluation of mutagenic potential.
Repeated dose toxicity Oral		
• 28- or 14-Day range-finding • 90-Day subchronic toxicity	OPPTS 870.3100 OECD 407 and 408	Evaluation of toxicity from repeated oral exposure; useful for identifying target organs.
Dermal		
• 21- or 28-Day range-finding • 90-Day subchronic toxicity	OPPTS 870.3200, 870.3250 OECD 410 and 411	Evaluation of toxicity from repeated dermal exposure; useful for estimating dermal absorption relative to oral route and identifying target organs.
Inhalation		
• 28- or 14-Day range-finding • 90-Day subchronic toxicity	OPPTS 870.3465 OECD 412 and 413	Evaluation of toxicity from repeated respiratory exposure; useful for identifying target organs.

Table 2: Synopses of Applicable U.S. EPA and OECD Toxicology Testing Guidelines

Toxicology Study Type	Test Guidelines	Value Added for Study Type
Repeated dose toxicity (continued)		
• Neurotoxicity	OPPTS 870.6200 OECD 424	Evaluation of toxicity to nervous system.
• Immunotoxicity	OPPTS 870.7800	Evaluation of toxicity to immune system.
• Reproduction	OECD 422	Initial evaluation of repeated dose, reproductive and developmental toxicity potential.
Endocrine disruption screening battery		
• *In vitro* screening	None available.	Initial evaluation; endocrine effects may be predictive for reproductive, neurologic, immunologic or carcinogenic activity.
• *In vivo* screening	None available.	Second tier evaluation; endocrine effects may be predictive for reproductive, neurologic, immunologic or carcinogenic activity.
Metabolism and pharmacokinetics (toxicokinetics)	OPPTS 870.7485 OPPTS 870.8500 OECD 417	Useful for determining the extent of body uptake from various exposure routes, distribution in the body, and elimination; for setting dose levels for chronic studies; and for extrapolating data from animals to humans.
Developmental toxicity	OPPTS 870.3700 OECD 414	Evaluation of toxicity to developing fetus.
Developmental neurotoxicity	OPPTS 870.6300	Evaluation of toxicity to developing nervous system of fetus.
Reproduction and fertility	OPPTS 870.3800 OECD 415 and 416	Evaluation of toxicity to reproduction using a one- or two-generation study design.
Chronic toxicity and carcinogenicity	OPPTS 870.4100, 870.4200 and 870.4300 OECD 451, 452 and 453	Evaluation of toxicity from lifetime exposure; useful for identifying target organs.
Mechanistic research	None available.	Research conducted in order to develop an understanding of how and why a specific type of toxic effect is manifested.

Table 2: Synopses of Applicable U.S. EPA and OECD Toxicology Testing Guidelines (continued)

In order to make reliable estimates of the potential toxicity of new chemicals, it is necessary to develop a database that provides a broad evaluation of the effects of the chemical on biological systems. The proposed strategy, outlined in Table 3, consists of four levels of experimentation. The lowest tier, Level 1, requires no animal studies. It serves to collect all of the available data that can be generated from theoretical evaluations and *in vitro* studies, as well as data that can be

derived from the literature on related chemicals. The second tier of testing, Level 2, involves the

Level 1 – Preliminary Evaluation
• Establish chemical identity;
• Estimate physical-chemical properties of chemical;
• Analyze structure of chemical; use expert systems or expert judgment to identify potential toxicologically active substructures; identify related chemicals;
• Develop toxicological profile using SAR and *in vitro* toxicity testing methods;
• Evaluate genetic toxicity using *in vitro* bacterial gene mutation assay;
• Collect toxicology data for related chemicals through database searching;
• Evaluate preliminary toxicology data and prepare a statement of needs for toxicity testing.

Level 2 – Basic Toxicology Studies
• Acute toxicity (oral, dermal and/or inhalation);
• Skin and eye irritation;
• Skin sensitization;
• Genetic toxicity *in vitro* (*in vitro* mammalian cell cytogenetics and/or *in vitro* mammalian cell gene mutation);
• 14-, 28- Day repeated dose toxicity (range-finding);
• Endocrine disruption *in vitro* screening battery;
• Evaluate Level 2 toxicity data and reach consensus opinion on Level 3 data needs.

Level 3 – Intermediate Toxicology Studies
• Metabolism and toxicokinetics;
• Additional genetic toxicity testing (*in vivo* micronucleus);
• 90-Day subchronic toxicity, possibly including neurotoxicity, immunotoxicity and reproductive toxicity evaluations;
• Developmental toxicity;
• Endocrine disruption *in vivo* screening battery;
• Evaluate Level 3 toxicity data and reach consensus opinion on Level 4 data needs.

Level 4 – Advanced Toxicology Studies
• Chronic toxicity/carcinogenicity;
• Two-generation reproductive toxicity;
• Developmental neurotoxicity;
• Evaluate Level 4 toxicity data and reach consensus opinion on data needs for risk assessment.

Risk Assessment
• Estimate virtually safe dose (VSD) for long-term exposures to chemical;
• Determine estimated human dose (EHD) to chemical under expected use conditions;
• Determine ratio of EHD to VSD and make recommendations for safe management of chemical.

Table 3: Overview of Data Development Strategy for New Chemicals standard short-term tests

standard for toxicity, including acute toxicity, eye and skin irritation and other evaluations. The third tier of proposed testing, Level 3, extends the database to subchronic toxicity endpoints and investigates the kinetics and metabolism of the chemical in the test animal. Level 4 studies are long term animal studies for chronic toxicity, carcinogenicity and reproductive toxicity. At each level of testing, several specific testing activities are proposed. It is not the intent that all tests must be performed at each level. Toxicity testing has many characteristics of a decision tree process. The next tests selected are determined, to a large extent, by the results of the previous test. Even standard testing procedures may be modified if particular issues need to be addressed. The specific tests proposed at each testing level are described in more detail in the following sections along with proposed criteria that define the levels of toxicological concern generated by the results of each test.

Establishing Levels of Toxicological Concern to Support Decision-Making Based on Health Hazard Data

For each relevant toxicological finding observed in the proposed testing strategy described in this report, criteria are proposed to establish a level of toxicological concern. Available toxicity data are reviewed and used to place each chemical into one of the following three categories: "High Concern", "Medium Concern" or "Low Concern" for oral, dermal and inhalation exposure. In cases where there are data available for a single route of exposure, an attempt should be made to extrapolate data to other relevant routes of exposure. In cases where there are no data available for a chemical by any route of administration or exposure, data for a close structural analog should be considered. Human toxicity data for closely related chemicals should be considered on a case-by-case basis to aid in establishing the overall level of concern for a chemical. Because the data set for a new chemical may vary both qualitatively and quantitatively, it may be appropriate to adjust the overall level of concern based on the level of confidence in the data. It is recommended that chemicals with toxicological classification of high concern for any endpoint require immediate action, those chemicals with toxicological classifications of low concern for all endpoints require no action, and chemicals with toxicological classifications of medium concern require case-by-case assessment using expert professional judgement to determine what actions are required.

Mechanistic Studies

Mechanistic research helps to explain how a chemical produces an adverse effect in laboratory animals or humans. Sometimes mechanistic research may take the form of more detailed metabolism and toxicokinetic studies. However, in the last few years, mechanistic research in toxicology has become increasingly focused on molecular mechanisms of action. Development of a fruitful mechanistic research program often requires involvement of external experts, particularly those from academia.

Mechanistic research may be considered at any point in the development of a new chemical if serious adverse systemic toxicity is suggested by toxicological studies or if analysis of structure-activity relationships (SAR) suggests structural alerts. If the mechanism of action can be identified in animal models, then it becomes feasible to determine whether the mechanism and the

subsequent response are likely to be expressed in humans. In most cases, mechanistic research is not pursued until significant toxicological data is developed, usually in Level 2 and/or Level 3 studies.

LEVEL 1 - EVALUATION OF AVAILABLE INFORMATION AND SCREENING EVALUATIONS

The objective of the Level 1 activities is to gather together readily available data relevant to evaluating the toxicity of the chemical of interest. These data are collected from a multitude of sources and form the basis for the initial review of the potential toxicological hazards associated with the use of the new material. To act as stewards for the development of the toxicological assessment, a small internal chemical steering committee should be formed to track and manage the evaluation process. This committee could consist of as few members as a single individual, if he/she is fully qualified and has adequate experience in toxicity testing. Under the direction of the chemical steering committee, the Level 1 activities are conducted and the information collected is entered into a toxicity database for easy reference. The following data are required for initial evaluation of the chemical.

Establish Chemical Identity

Chemical structure is the necessary basis for new chemical identity. In rare circumstances, a Chemical Abstracts Service registry number (CAS RN) may be available. Analytical methods, such as mass spectrometry, infrared spectroscopy, multiple nuclei nuclear magnetic resonance spectroscopy and elemental analysis may provide valuable information to establish chemical structure.

Several questions facilitate the characterization of the chemical of interest:

- Has the structure of the new chemical been determined?
- Have all significant components or impurities (to within 0.1%) been identified?
  - The amount and toxicity of components and impurities are important because either may affect the overall health hazard properties of a chemical.
- Has the information on which characterization is based been obtained on the form of the chemical that is representative of the substance to which there may be occupational exposures?
  - This is particularly important if impurities or minor components can significantly alter the health hazard effects of a chemical.
- Can the new chemical be placed in a chemical category based on functionality or reactive moiety or placed in a class of related substances?
- Is the new chemical similar structurally to other well-characterized chemical substances, i.e. close structural analogs?
  - Structure-activity relationships (SAR) may provide a basis for making judgements about the potential health hazards of a chemical.

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Estimate/Determine Physical-Chemical Properties

Quantitative data for the physical-chemical properties (i.e. physical state, melting point, boiling point, vapor pressure, solubility, octanol-water partition coefficient, viscosity, etc.) are necessary for characterization of the chemical. These data may be obtained experimentally or by the use of computational chemistry tools if chemical structures and component composition are well known. Some assessment of the stability of the chemical under storage conditions and as a prepared stock solution is necessary to assure meaningful biological system dosing and testing results. These data are paramount to gain some perspective on the relevant toxicological issues. For example, if the vapor pressure is high, then there is an excellent chance for exposure to vapors via the inhalation route and this would be the dominant route of exposure. If the octanol-water partition coefficient is large, then accumulation in body fat becomes an important concern. Therefore, physical-chemical characterization is a key step in the evaluation of a new chemical.

Estimate Occupational Exposure

Since the main objective of this report is to provide a data development strategy for occupational health hazard evaluation of new chemicals, consideration of the applications of the new chemical is relevant at this stage. Several questions may need to be considered:

- Will the chemical be used solely as an intermediate that is chemically reacted to produce other substances or will it be used widely in Air Force field applications?
- Would exposure occur with the parent chemical or a degradation product or products that may vary due to storage as well as environmental release conditions?
- Could use and disposal lead to human exposure and/or environmental release of the parent chemical or a degradation product?
- If the chemical could be released to the environment, how would it be expected to partition among environmental media (soil, water, air)?
- What populations might be exposed to the parent chemical or a degradation product?

A theoretical analysis of the occupational exposure potential for the new chemical should be undertaken at this stage by industrial hygienists and biomedical engineers. An industrial hygienist will evaluate the physical characteristics of the facility where exposure is likely to occur; observe how the chemical or similar chemicals are handled, i.e. open or closed systems; determine the makeup of the population of potentially exposed workers; identify types of work assignments of potentially exposed workers; evaluate the physicochemical properties of the new substance, which aid in the identification of potential routes of worker exposure; conduct quantitative measures of exposure to chemicals with similar physical-chemical properties experimentally obtained by air sampling; and assess potential for accidental exposures. If the new chemical eventually will be manufactured and/or handled in relatively large volume, or if the health hazard evaluation identifies areas of concern, a more detailed occupational exposure assessment may be warranted when the chemical is deployed to the field by the biomedical engineers. This initial analysis will be used to identify the most likely route of exposure to the chemical following release and subsequent deposition sites for acute occupational and long term potential of environmental exposure.

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Develop Preliminary Toxicological Profile

Structure-activity relationships

Structure-activity relationships (SARs), based on scientific judgements by experienced toxicologists, may be used as an integral part of health hazard characterization. This approach relies on the toxicologist or chemist being able to fit the new chemical into a category of existing chemicals because of similarities in molecular structure or chemical functionality. In order for this approach to be of value, the existing category of chemicals or a close structural analog must have its own robust toxicology database. The uncertainty of correlated toxicity from the close structural analogs or the category to the new chemical must be recognized and, if possible, defined using both similarity and dissimilarity of chemical structure, functionality and reactivities at standard temperature and pressure (STP).

SAR analysis has been formalized and computerized for some health endpoints (i.e. in particular for cancer, mutagenicity and teratogenicity) and may be useful with appropriate recognition of the limitations of these programs. A review of the various computerized SAR programs available commercially is beyond the scope of this document, but this subject has been reviewed recently (Dearden *et al.* 1997).

The U.S. EPA has grouped chemical substances with similar physical-chemical, structural and toxicological properties into working categories. Additional candidate categories for the EPA's new chemical review process are proposed by the TSCA New Chemicals Program (NCP) staff based on available data and experience of reviewing PMNs on related substances. These groupings enable the user of the NCP Chemical Categories guidance document to benefit from the accumulated data and decisional precedents within the EPA new chemicals review process since 1987, in order to identify areas of health hazard concern. Currently, there are 51 chemical categories listed in the table of contents of the EPA document, the detailed summaries of which may be found at URL: http://www.epa.gov/opptintr/newchms/chemcat.htm.

Close structural analogs may provide data for occupational exposure and/or data for health hazard evaluation from occupational experience or epidemiology studies. For close structural analogs that have been used for several years, relevant information may be gained from historical experience with human exposure based on normal handling and accidental larger level exposures. For many existing chemicals with years of widespread industrial use, no adverse health effects have been observed. On the other hand, in some cases of overexposure or where unexpected toxicity was discovered, adverse effects in occupational populations have occurred. When they are available, retrospective (or case-control) epidemiological studies for close structural analogs may provide insight as to the potential for certain health effects by the new chemical.

In vitro cytotoxicity screening tests

In many cases, SAR can not be performed without a high degree of uncertainty based on insufficient availability of human health hazard or animal toxicology data on close structural analogs of the new chemical to conduct a preliminary health hazard characterization. In such

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instances, *in vitro* toxicity screening tests may be advisable to develop the data necessary for a preliminary health hazard characterization for the new chemical. The role of *in vitro* toxicity testing in chemical hazard characterization has not been formalized by U.S. regulatory agencies. However, a wide range of *in vitro* screening tests have been developed, appropriate for the correlation to the toxicology endpoints used in *in vivo* testing proposed at Level 2, i.e. acute oral, dermal and/or inhalation toxicity; skin and eye irritation; skin sensitization; genetic toxicity; short-term repeated dose toxicity; and endocrine disruption screening. Cytotoxicity assays are promoted as suitable models for screening for human toxicity. A listing of proposed *in vitro* methods that may be used at Level 1 to generate data predictive of Level 2 toxicology results and human health hazard potential are the following:

- Acute toxicity → Cytotoxicity assays using cultured cells (Seiberts 1996)

- Skin irritation → *In vitro* skin corrosivity/irritation testing - CORROSITEX™ (Botham *et al.* 1994)

- Eye irritation → *In vitro* eye corrosivity/irritation testing - EYETEX™ or HET-CAM (Balls *et al.* 1998)

Evaluate Genetic Toxicity

In vitro Genotoxicity

Due to the high level of concern for knowledge regarding chemicals that may cause cancer, an early evaluation of the potential for a chemical to cause mutations, i.e., an alteration of the genetic information stored in the DNA of a cell, is highly recommended. The bacterial reverse mutation assay, often referred to as the Ames assay, is a valuable early screen for genotoxicity. The bacterial reverse mutation test typically costs $5,000, requires approximately three months from initiation to final report, and utilizes no animals. The purpose, initial considerations and principle of the test method are described in OPPTS 870.5100 "Bacterial Reverse Mutation Test" (edited excerpts follow).

> **Purpose.** The bacterial reverse mutation test uses a bacterium single DNA base mutation with an essential, normally produced protein that generates a specific amino-acid culture of selected strains of *Salmonella typhimurium (S. typhimurium)* and *Escherichia coli (E. coli)* requiring amino acid supplementation to grow. The strains have mutational regeneration involving substitution, addition or deletion of one or a few DNA base pairs. The principle of this bacterial reverse mutation test is that it detects mutations that revert. These pre-existing mutations present in the test strains and restore the functional capability of the bacteria to synthesize the essential amino acid. Following chemical exposure, the revertant bacteria are detected by their ability, to grow by transfer to growth media absent the amino acid required by the parent test strain.

> Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumor suppressor genes of somatic cells

are involved in tumor formation in humans and experimental animals. The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the test strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems as well as enhancement of error-prone DNA repair processes. The specificity of the test strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large database of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physical-chemical properties, including volatile compounds.

Initial considerations. The bacterial reverse mutation test utilizes prokaryotic cells (bacteria), which differ from mammalian cells in such factors as glycoslyated cell walls rather than lipid protein membranes affecting constitutive and xenobiotic uptake, metabolism, chromosome structure and DNA repair processes and gene expression and protein processing. Tests conducted *in vitro* generally require the use of an exogenous source such as the membrane bound enzymes of liver for metabolic activation. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. The test, therefore, does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals.

The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. An extensive data base has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests. There are examples of mutagenic agents, which are not detected by this test; reasons for these shortcomings can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability. On the other hand, factors that enhance the sensitivity of the bacterial reverse mutation test can lead to an overestimation of mutagenic activity. The bacterial reverse mutation test may not be appropriate for the evaluation of certain classes of chemicals, for example highly bactericidal compounds (e.g. certain antibiotics) and those that are thought (or known) to interfere specifically with the mammalian cell replication system (e.g. some topoisomerase inhibitors and some nucleoside analogues). In such cases, mammalian mutation tests may be more appropriate.

Although many compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute. It is dependent on chemical class, and there are carcinogens that are not detected by this test because they act through other, nongenotoxic mechanisms or mechanisms absent in bacterial cells.

Principle of the test method. Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium. In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal

medium. For both techniques, after 2 or 3 days of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates.

Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method, the preincubation method, the fluctuation method and the suspension method. Suggestions for modifications for the testing of gases or vapors have been described.

The procedures described in this guideline pertain primarily to the plate incorporation and preincubation methods. Either of them is acceptable for conducting experiments both with and without metabolic activation. Some compounds may be detected more efficiently using the preincubation method. These compounds belong to chemical classes that include short chain aliphatic nitrosamines, divalent metals, aldehydes, azodyes and diazo compounds, pyrollizidine alkaloids, allyl compounds and nitro compounds. It is also recognized that certain classes of mutagens are not always detected using standard procedures such as the plate incorporation method or preincubation method. These should be regarded as "special cases" and it is strongly recommended that alternative procedures should be used for their detection. The following "special cases" could be identified (together with examples of procedures that could be used for their detection): azodyes, diazo compounds, gases, volatile chemicals, and glycosides. A deviation from the standard procedure needs to be scientifically justified.

The data provided by the bacterial reverse mutation assay allows for an early evaluation of the mutagenic potential of the chemical.

Collect Toxicity Data on Related Chemicals

Given the complexity of molecular structures, selection of structurally-related analogs to the new chemical is usually based on expert judgement. Valid analogs should have close three-dimensional or space filling structural similarity and the same functional groups. Structurally-related chemicals are likely to have similar physical-chemical, environmental and toxicological properties or follow a predictable pattern of effects. The similarities may be based on the following list adopted from the US EPA (http://www.epa.gov/chemrtk/categuid.htm):

- A common functional group (e.g. aldehyde, epoxide, ester, etc.); or
- The likelihood of common precursors and/or breakdown products, via physical or biological processes, which result in structurally similar chemicals; and
- An incremental and constant change across a group of structurally related chemicals.

Having identified structurally related chemicals, it is necessary to search the literature for relevant toxicological information. When conducting literature searches it is recommended to proceed in three phases. In the first phase, the ***IUCLID, ***TSCATS and ***CHEMID databases are searched for all relevant references to toxicological information on the identified analog chemicals. The IUCLID and TSCATS databases should identify numerous studies that provide toxicological data for analog chemicals that could be applied to the new chemical. CHEMID is

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useful in that it identifies other databases with pertinent studies.

The next phase of the literature search is to review relevant information in the ***RTECS and ***HSDB databases to identify additional studies. RTECS and HSDB are particularly useful because these databases are data files (rather than bibliographic databases) that contain data by chemical and study type.

The final phase of the search strategy involves searching of specific databases and/or the uses of specific strategies to locate studies of a particular type. For example, if the database for an analog of a new chemical appeared to be missing reproductive or developmental toxicity study data following the initial search, ***DART and ***ETICBACK can be searched because these databases specifically include studies with reproductive and developmental endpoints. If mutagenicity study data were missing, then Cancerlit, ***CCRIS, ***EMIC, ***EMICBACK and GENE-TOX could be searched. If appropriate data could not be identified using the aforementioned databases, Toxline and Medline files can be searched using a strategy that would specifically identify appropriate studies for the endpoint of interest.

Evaluate Preliminary Toxicological Data

At the completion of the Level 1 activities the following information is available: physical-chemical properties, estimate of exposure circumstances, preliminary toxicological data (SAR and *in vitro* data) and toxicological profiles on structurally related chemicals. An evaluation of these data by the chemical steering committee will establish an overall perception of the potential toxicological concerns with the use of the new chemical under operational conditions. Advice of the committee should address specific data needs for hazard assessment with the intention of developing a database for the new chemical sufficiently robust to perform risk assessment adequately and deal with risk management issues.

The chemical steering committee should provide specific guidance for the type of toxicological data needed to satisfy the requirements of all stakeholders. The proposed testing strategy, based on the multilevel tier approach described in this report, should identify the most likely toxicological issues related to the particular chemical and its expected use. Taking into consideration the expected route of exposure and potential toxicological properties, a checklist of recommended toxicity tests should be prepared identifying tests and crosslisting tests with anticipated toxicological issues. For example, if evaluation of the toxicity of closely related structural analogs suggests that developmental toxicity may be a concern, then the testing strategy should emphasize developmental toxicity testing. The following sections discuss the candidate toxicity tests suggested for extension of the toxicity database at Level 2.

LEVEL 2 - BASIC TOXICOLOGY STUDIES

Level 2 toxicology studies provide the first *in vivo* toxicity data required to screen for potential human health hazards of the chemical of interest. The amount of data that need to be developed depends somewhat on the strategy developed as a result of the Level 1 evaluation. However, generally speaking, a minimum base data set should be developed for new chemicals that are

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expected to be used in Air Force applications. This minimum data set includes acute toxicity testing by one relevant route of exposure, skin and eye irritation testing, skin sensitization testing and additional *in vitro* gene mutation testing. The other toxicity tests suggested for Level 2 studies, 28 day repeated dose studies and the endocrine disruptor screening should be conducted at the discretion of the chemical steering committee using consideration of longer low-level exposure based on the chemicals use profile. The reader is referred to the EPA Health Effects Test Guidelines (U.S. EPA 1996) and OECD Guidelines for Testing of Chemicals (1998) for the specific protocol requirements of Level 2 studies.

An acute toxicity testing battery (oral, dermal and/or inhalation; skin and eye irritation; skin sensitization) is conducted to identify any hazards that may arise from handling a new chemical in cases where there might be short-term, relatively high level exposures. In an occupational setting, inhalation and skin and eye contact are the most common routes of exposure. Nonetheless, most acute toxicity testing batteries include an acute oral toxicity study. These studies help to define safe levels of short-term exposure or personal protection equipment needs, as well as to form the basis for the initial selection of doses for short-term repeated dose studies.

Acute Toxicity

A number of classification schemes that have been developed by regulatory authorities in North America and Europe (i.e. U.S. EPA, Occupational Safety and Health Administration (OSHA), Consumer Product Safety Commission (CPSC), Department of Transportation (DOT), ***WHMIS and the ***EU DSD) are available for categorizing chemicals based on acute oral, dermal and inhalation toxicity. These classification schemes were utilized to assist in the development of guidelines to classify chemicals of interest to the Air Force as to their level of toxicological concern. In these guidelines, the values used to set boundaries for each category of concern are expressed on a mg/kg basis for acute oral and dermal toxicity and on a mg/l basis for acute inhalation toxicity.

Acute oral

The acute oral toxicity study typically costs $5,000, requires approximately three months from initiation to final report and utilizes between five and 30 rats. The purpose, approaches and principle of the test method are described in OPPTS 870.1100 "Acute Oral Toxicity" (edited excerpts follow).

> **Purpose.** In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards likely to arise from short-term exposure by the oral route. Data from an acute oral study may serve as a basis for classification and labeling. It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance. An evaluation of acute oral toxicity data should include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioral and

clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

Conventional acute oral toxicity test--principle of the test method. The test substance is administered orally by gavage in graduated doses to several groups of experimental animals, one dose being used per group. The doses chosen may be based on the results of a range finding test. Subsequently, observations of effects and deaths are made. Animals that die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied. This guideline is directed primarily to studies in rodent species but may be adapted for studies in nonrodents. Animals showing severe and enduring signs of distress and pain may need to be euthanized. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

Acute Dermal

The acute dermal toxicity study typically costs about $5,000, requires approximately three months from initiation to final report, and utilizes between five and 30 rats. The purpose, approaches and principle of the test methods are described in OPPTS 870.1200 "Acute Dermal Toxicity" (edited excerpts follow).

Purpose. In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is useful where exposure by the dermal route is likely. It provides information on health hazards likely to arise from short-term exposure by the dermal route. Data from an acute dermal study may serve as a basis for classification and labeling. It is an initial step in establishing a dosage regimen in subchronic and other studies and may provide information on dermal absorption and the mode of toxic action of a substance by this route. An evaluation of acute dermal toxicity data should include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

Conventional acute dermal toxicity test-principle of the test method. The test substance is applied dermally in graduated doses to several groups of experimental animals, one dose being used per group. Doses and treatment of animals is similar to that discussed above for oral dosing.

Acute Inhalation

The acute inhalation toxicity study typically costs $20,000, requires approximately three months from initiation to final report, and utilizes between ten and 40 rats. The purpose, approaches and principle of the test method are described in OPPTS 870.1300 "Acute Inhalation Toxicity" (edited excerpts follow).

Purpose. Determination of acute inhalation toxicity is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance that may be inhaled, such as a gas, volatile substance, or aerosol particle. It provides information on health hazards likely to arise from short-term exposure by the inhalation route. Data from an acute inhalation study may serve as a basis for classification and labeling. It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance. An evaluation of acute inhalation toxicity data should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

Conventional acute inhalation toxicity test--principle of the test method. Several groups of experimental animals are exposed to the test substance in graduated concentrations for a defined period, one concentration being used per group. When a vehicle other than water is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group should be used when historical data are not available or adequate to determine the acute inhalation toxicity of the vehicle. Subsequently, observations of effects and death are made. Doses and treatment of animals is the same as discussed for oral testing above.

The following guidelines are used to place a chemical into one of three categories of concern based on its approximate acute LD_{50} or LC_{50} (lethal dose or lethal concentration, respectively, for 50% of exposed animals) value.

High Concern:

- Oral LD_{50} < 50 mg/kg;

- Dermal LD_{50} < 200 mg/kg;

- Inhalation 4-hour LC_{50} < 0.3 mg/l;

- Inhalation 1-hour LC_{50} < 1.2 mg/l.

Medium Concern:

- Oral LD_{50} ≥ 50 mg/kg but < 500 mg/kg;

- Dermal LD_{50} ≥ 200 mg/kg but < 1000 mg/kg;

- Inhalation 4-hour LC_{50} ≥ 0.3 mg/l but < 3.0 mg/l;

- Inhalation 1-hour LC_{50} ≥ 1.2 mg/l but < 12.0 mg/l.

Low Concern:

- Oral LD_{50} ≥ 500 mg/kg;

- Dermal $LD_{50} \geq 1000$ mg/kg;

- Inhalation 4-hour $LC_{50} \geq 3.0$ mg/l;

- Inhalation 1-hour $LC_{50} \geq 12.0$ mg/l.

The US EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemical exposure while preserving its ability to make reasonable judgments about safety (edited excerpts from Approaches as described in OPPTS 870.1100, 870.1200 and 870.1300):

(i) For estimation of acute toxicity, EPA generally supports the use of appropriate alternative test protocols when available. Thus, for example, acute oral toxicity testing may be performed using the Fixed Dose Method, the Acute Toxic Method or the Up-and-Down Method. The fixed dose procedure is a refinement of the traditional acute oral test that employs nonlethal endpoints. In contrast, the acute toxic method and up-and-down procedures estimate lethality within a dose range and as a point estimate, respectively, and reduce animal usage in comparison to the "traditional" LD_{50} test.

(ii) When acute toxicity data is required, a limit test may be considered. If rodents are used for evaluating oral toxicity, a limit dose of at least 2000 mg/kg of body weight may be administered to a single group of five males and five females using the procedures described in the EPA guidelines. If no lethality is demonstrated, no further testing for acute oral toxicity is needed. If compound-related mortality is produced in the limit test, further study using the methods described above may need to be considered.

(iii) For acute dermal toxicity, a limit dose of at least 2000 mg/kg body weight may be administered as described in the guidelines. For inhalation studies, a single group of five males and five females is exposed to 2 mg/l for 4 hours, or where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration.

(iv) In order to minimize the need for animal testing, the US EPA encourages the review of existing acute toxicity information on mixtures that are substantially similar to the mixture under investigation. In certain cases it may be possible to glean enough information to make preliminary hazard evaluations that may reduce the need for further animal testing.

Primary Skin and Eye Irritation

Skin Irritation

The primary skin irritation study typically costs $1,000, requires approximately three months from initiation to final report, and utilizes between one and three rabbits. The purpose, principle of test method and initial considerations are described in OPPTS 870.2500 "Acute Dermal Irritation" (edited excerpts follow).

> **Purpose.** Determination of the irritant and/or corrosive effects on skin of mammals is useful in the assessment and evaluation of the toxic characteristics of a substance where exposure by the dermal route is likely. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance.
>
> **Principle of the test method.** The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control (except when severe irritation/corrosion is suspected and the stepwise procedure is used). The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed, but need not exceed 14 days.
>
> **Initial considerations.** Strongly acidic or alkaline substances, for example with a demonstrated pH of 2 or less, or 11.5 or greater, need not be tested for primary dermal irritation, owing to their predictable corrosive properties.
>
> It is unnecessary to test materials that have been shown to be highly toxic (LD_{50} less than 200 mg/kg) by the dermal route or have been shown not to produce irritation of the skin at the limit test dose level of 2000 mg/kg body weight.
>
> It may not be necessary to test *in vivo* materials for which corrosive properties are predicted on the basis of results from well validated and accepted *in vitro* tests. If an *in vitro* test is performed before the *in vivo* test, a description or reference to the test, including details of the procedure, must be given together with results obtained with the test and reference substances.
>
> It may not be necessary to test materials for which corrosive potential is predicted from structure-activity relationships.

Eye Irritation

The primary eye irritation study typically costs $1,000, requires approximately three months from initiation to final report, and utilizes between one and three rabbits. The purpose, principle of the

test method and initial considerations are described in OPPTS 870.2400 "Acute Eye Irritation" (edited excerpts follow).

Purpose. In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosive effects on eyes of mammals is an important initial step. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the eyes and associated mucous membranes to the test substance.

Principle of the test method. The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals; the untreated eye is used to provide control information. The degree of irritation/corrosion is evaluated and scored at specified intervals and is fully described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed. The period of observation should be at least 72 hours, but need not exceed 21 days. Animals showing severe and enduring signs of distress and pain may need to be euthanized.

Initial considerations. Strongly acidic or alkaline substances, for example, with a demonstrated pH of 2 or less or 11.5 or greater, need not be tested owing to their predictable corrosive properties. Buffer capacity should also be taken into account.

Materials that have demonstrated definite corrosion or severe irritation in a dermal study need not be further tested for eye irritation. It may be presumed that such substances will produce similarly severe effects in the eyes.

Results from well validated and accepted *in vitro* test systems may serve to identify corrosives or irritants such that the test material need not be tested *in vivo*.

The severity and reversibility of irritation serve as the basis for establishing levels of concern for primary skin and eye irritation.

High Concern: Corrosive.

Medium Concern: Severe reversible irritation.

Low Concern: Slight to moderate reversible irritation.

Skin Sensitization

The skin sensitization study typically costs $10,000, requires approximately three months from initiation to final report, and utilizes between 15 and 30 guinea pigs. The purpose, principle of the test method and test procedures are described in OPPTS 870.2600 "Skin Sensitization" (edited excerpts follow).

Purpose. Determination of the potential to cause or elicit skin sensitization reactions (allergic contact dermatitis) is an important element in evaluating the toxicity of a substance. Information derived from skin sensitization tests serves to identify possible hazards to a population exposed repeatedly to a test substance. The test selected should identify substances with significant allergenic potential and minimize false negative results.

Principle of the test method. Following initial exposure to a test substance, the animals are subjected, after a period of not less than one week, to a challenge exposure with the test substance to establish whether a hypersensitive state has been induced. Sensitization is determined by examining the reaction to the challenge exposure and comparing this reaction with that of the initial induction exposure. The test animals are initially exposed to the test substance by intradermal and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (the induction period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure is compared with that demonstrated by control animals that undergo sham treatment during induction and then receive the challenge exposure.

Test procedures. Any of the following test methods are considered to be acceptable.

- Buehler test.
- Guinea-pig maximization test (GPMT).
- Other.
  - Open epicutaneous test.
  - Maurer optimization test.
  - Split adjuvant technique.
  - Freund's complete adjuvant test.
  - Draize sensitization test.

The GPMT of Magnusson and Kligman, which use adjuvant, and the nonadjuvant Buehler test are preferred over other methods. Although strong preference is given to either the GPMT or the Buehler test, it is recognized that other tests may provide useful results. If other tests are used, the tester should provide justification/reasoning for their use, methods and protocols must be provided, and each test should include a positive and a negative control group.

Recently, an alternative sensitization test has been validated by the Interagency Coordinating Committee for the Validation of Alternative Methods, a US federal government committee. This test is the local lymph node assay (Kimber, 1997) and has been accepted by several government agencies for evaluating skin sensitization. The assay consists of repeated application of the test chemical to mice ears (three consecutive days). After 5 days, the mice receive an injection of radiolabelled thymidine and five hours later the lymph nodes draining the ears are removed and assayed for accumulated radioactivity. A 3 fold increase in radioactivity above controls is considered a positive response. This assay uses fewer animals and is less stressful than the traditional tests for immunological sensitivity.

The levels of concern for skin and respiratory sensitization are established based on the criteria listed below.

High Concern: Clear evidence of sensitization in animal studies and evidence that structural analogs of the chemical are sensitizers.

Medium Concern: Clear evidence of sensitization in animal studies or evidence that structural analogs of the chemical are sensitizers.

Low Concern: Equivocal evidence of sensitization in animal studies or weak evidence that structural analogs of the chemical are sensitizers.

Genetic Toxicity

Initial data on the genotoxicity of the chemical of interest was developed in the Level 1 evaluation using the *in vitro* bacterial gene mutation assay. If additional information is required, additional *in vitro* genetic toxicity studies can be undertaken (*in vitro* mammalian cell cytogenetics or *in vitro* mammalian cell gene mutation) to evaluate further the mutagenic potential of chemicals. *In vitro* assays are an efficient approach to the initial assessment of mutagenic potential. The *in vitro* mammalian cell cytogenetics assay is most commonly used, but, in the case of a positive *in vitro* bacterial gene mutation test, an *in vitro* mammalian cell gene mutation is often performed. If these *in vitro* tests are uniformly negative, there is good reason to believe that the chemical is not mutagenic.

In vitro Mammalian Cell Cytogenetics

The *in vitro* mammalian chromosome aberration test typically costs $20,000, requires approximately three months from initiation to final report, and utilizes no animals. The purpose, initial considerations and principle of the test method are described in OPPTS 870.5375 "*In vitro* Mammalian Chromosome Aberration Test" (edited excerpts follow).

> **Purpose.** The purpose of the *in vitro* chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. However, this guideline is not designed to measure numerical aberrations and is not routinely used for that purpose. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour-suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals.

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The *in vitro* chromosome aberration test may employ cultures of established cell lines, cell strains or primary cell cultures. The cells used are selected on the basis of growth ability in culture, stability of the karyotype, chromosome number, chromosome diversity, and spontaneous frequency of chromosome aberrations.

Initial considerations. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions. Care should be taken to avoid conditions, which would lead to positive results that do not reflect intrinsic mutagenicity and may arise from changes in pH, osmolality or high levels of cytotoxicity.

Principle of the test method. Cell cultures are exposed to the test substance both with and without metabolic activation. At predetermined intervals after exposure of cell cultures to the test substance, they are treated with a metaphase-arresting substance (e.g. Colcemid® or colchicine), harvested, stained, and metaphase cells are analysed microscopically for the presence of chromosome aberrations.

In vitro Mammalian Cell Gene Mutation

The *in vitro* mammalian cell gene mutation test typically costs $20,000, requires approximately three months from initiation to final report, and utilizes no animals. The purpose (Introduction), initial considerations and principle of the test method are described in OPPTS 870.5300 "*In vitro* Mammalian Cell Gene Mutation Test" (edited excerpts follow).

Introduction. The *in vitro* mammalian cell gene mutation test can be used to detect gene mutations induced by chemical substances. Suitable cell lines include L5178Y mouse lymphoma cells, the CHO, AS52 and V79 lines of Chinese hamster cells, and TK6 human lymphoblastoid cells. In these cell lines the most commonly-used genetic endpoints measure mutation at thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HPRT), and a transgene of xanthine-guanine phosphoribosyl transferase (XPRT). The TK, HPRT and XPRT mutation tests detect different spectra of genetic events. The autosomal location of TK and XPRT may allow the detection of genetic events (e.g. large deletions) not detected at the HPRT locus on X-chromosomes.

Initial considerations. In the *in vitro* mammalian cell gene mutation test, cultures of established cell lines or cell strains can be used. The cells used are selected on the basis of growth ability in culture and stability of the spontaneous mutation frequency. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions. Care should be taken to avoid conditions that would lead to results not reflecting intrinsic mutagenicity. Positive results that do not reflect intrinsic mutagenicity may arise from changes in pH, osmolality or high levels of cytotoxicity.

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not

a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through other, nongenotoxic mechanisms or mechanisms absent in bacterial cells.

Principle of the test method. Cells deficient in thymidine kinase (TK) due to the mutation $TK^+/- \rightarrow TK-/-$ are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). Thymidine kinase proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain thymidine kinase, are not. Similarly, cells deficient in HPRT or XPRT are selected by resistance to 6-thioguanine (TG) or 8-azaguanine (AG). The properties of the test substance should be considered carefully if a base analogue or a compound related to the selective agent is tested in any of the mammalian cell gene mutation tests. For example, any suspected selective toxicity by the test substance for mutant and nonmutant cells should be investigated. Thus, performance of the selection system/agent must be confirmed when testing chemicals structurally related to the selective agent.

Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a suitable period of time and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. Cytotoxicity is usually determined by measuring the relative cloning efficiency (survival) or relative total growth of the cultures after the treatment period. The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each selected locus and cell type, to allow near-optimal phenotypic expression of induced mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the cloning efficiency (viability). After a suitable incubation time, colonies are counted. The mutant frequency is derived from the number of mutant colonies in selective medium and the number of colonies in nonselective medium.

The level of concern for mutagenicity is assigned as described below based on the weight of evidence from the battery of mutagenicity tests.

High Concern: Positive in all *in vitro* tests and QSAR analysis.

Medium Concern: Several positive assay results but overall conflicting findings across assays in test battery.

Low Concern: Negative in all *in vitro* tests and QSAR analysis.

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens; however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and

there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through mechanisms other than direct DNA damage. However, from a practical point of view, if all evaluations indicate that the chemical is positive for mutagenicity, the likelihood that the chemical will be carcinogenic in an *in vivo* chronic study is high. Therefore, a careful evaluation of the future use of the chemical should be undertaken.

Repeated Dose Toxicity

Repeated dose toxicity is used for compounds known to be used commonly in manufacturing and occupational practices, or those with either medium or high concern following acute in vitro/in vivo testing.

28-Day repeated dose toxicity studies (oral, dermal or inhalation) are conducted as dose range-finding studies for 90-day subchronic toxicity studies. Usually, doses are chosen to produce frank toxicity at the highest dose level and no effects at the lowest dose. Study designs employed for range-finding studies are similar to those used for 90-day subchronic toxicity studies, except fewer animals are used, fewer organs are evaluated at necropsy and histopathology usually is not performed. Short-term repeated dose studies are important tools for the early identification of major target organ effects and the initial assessment of the dose-response relationship for a chemical.

Repeated Dose Oral

Repeated dose 28-day oral toxicity studies typically cost $35,000 to $60,000, require approximately three to four months from initiation to final report, and utilize between 40 and 80 rats. The initial considerations and principle of test method are described in OECD Guideline 407 "Repeated Dose 28-day Oral Toxicity Study in Rodents" (edited excerpts follow).

> **Initial Considerations.** In the assessment and evaluation of the toxic characteristics of a chemical, the determination of oral toxicity using repeated doses may be carried out after initial information on toxicity has been obtained by acute testing. This study provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time. The method comprises the basic repeated dose toxicity study that may be used for chemicals on which a 90-day study is not warranted (e.g. when the production volume does not exceed certain limits) or as a preliminary to a long-term study. The duration of exposure should normally be 28 days although a 14-day study may be appropriate in certain circumstances; justification for use of a 14-day exposure period should be provided.
>
> The Guideline places more emphasis on neurological effects as a specific endpoint, and the need for careful clinical observations of the animal are stressed so as to obtain as much information as possible. The method should identify chemicals with neurotoxic potential, which may warrant further in-depth investigation of this aspect. In addition, the method may give an indication of immunological effects and reproductive organ toxicity.

Principle of the test method. The test substance is orally administered daily in graduated doses to several groups of experimental animals, one dose level per group for a period of 28 days. During the period of administration the animals are observed closely, each day for signs of toxicity. Animals that die or are killed during the test are necropsied and, at the conclusion of the test, surviving animals are killed and necropsied.

Repeated Dose Dermal

Repeated dose 28- day dermal toxicity studies typically cost $45,000 to $60,000, require approximately three to four months from initiation to final report, and utilize between 40 and 80 rats. The purpose of the test method is described in OECD Guideline 410, "Repeated Dose Dermal Toxicity: 21/28-day Study" (edited excerpts follow).

> **Introduction, Purpose, Scope, Relevance, Application and Limits of test.** In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic dermal toxicity may be carried out after initial information on toxicity has been obtained by acute testing. It provides information on possible health hazards likely to arise from repeated exposures by the dermal route over a limited period of time.
>
> **Principle of the test method.** The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose per group, for a period of 28 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals that die during the test are necropsied, and, at the conclusion of the test, the surviving animals are sacrificed and necropsied.

Repeated Dose Inhalation

Repeated dose 28-day inhalation toxicity studies typically cost $70,000 to $90,000, require approximately four to five months from initiation to final report, and utilize between 40 and 80 rats. The purpose and principle of test method are described in OECD Guideline 412 "Repeated Dose Inhalation Toxicity: 28-day or 14-day Study" (edited excerpts follow).

> **Introduction, purpose, scope, relevance, application and limits of test.** In the assessment and evaluation of the toxic characteristics of an inhalable material, such as a gas, volatile substance or aerosol/particulate, determination of inhalation toxicity using repeated exposures may be carried out after initial information on toxicity has been obtained by acute testing. It provides information on health hazards likely to arise from repeated exposure by the inhalation route over a limited period of time. Hazards of inhaled substances are influenced by the inherent toxicity and by physical factors such as volatility and particulate size.
>
> **Principle of the test method.** Several groups of experimental animals are exposed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 28 days. Where a vehicle is used to help generate an appropriate concentration of the test substance in the atmosphere, a vehicle control group should be used. During the period of administration the animals are observed daily to

detect signs of toxicity. Animals that die during the test are necropsied, and, at the conclusion of the test, surviving animals are sacrificed and necropsied.

Short-term subchronic toxicity tests are considered as preliminary studies for the full 90-day subchronic test (see below). As such they are not designed to provide definitive toxicity results. However, during the conduct of the short-term subchronic toxicity test, adverse findings are expected at the highest dose. These data should be evaluated and, if considered relevant, the focus of later stages of the testing strategy modified appropriately. If adverse effects are observed at dosages significantly below the dose expected to produce frank toxicity, based on extrapolating results of acute toxicity testing, then the level of concern should be increased.

For subchronic (and chronic) toxicity, reported effects can be grouped into three general categories depending on whether they represent adaptive or nonspecific effects, effects indicative of target organ or systemic toxicity, or effects that are not relevant to human health. This distinction is made because adaptive or nonspecific effects likely will lead to a lower level of concern than a specific target organ or systemic effect noted at a comparable dose level. No level of concern is placed on effects that are not relevant to human health. Examples of effects that may be seen in the three general categories are listed below.

Adaptive and nonspecific effects:

- Reversible, adaptive changes, i.e. liver microsomal enzyme induction;

- Reversible changes directly related to the route of administration, i.e. gastrointestinal irritation;

- Slight decreases in body weight gain or food consumption;

- Nondebilitating clinical signs, such as unkempt appearance.

Target organ or clear systemic effects:

- Marked body weight effects;

- Behavioral, anatomic or clinical pathology changes;

- Evidence for proliferative tissue changes in subchronic toxicity studies that would suggest an increased potential for tumor development with longer-term exposure;

- Effects on survival;

- Evidence for neoplastic changes (more likely to be found in chronic toxicity studies).

Effects that are not relevant to human health:

- Effects that are secondary to nutritional imbalances caused by diet rejection or intake of the test substance at a dose level that exceeds metabolic capacity for that substance;

- Effects seen in laboratory animals for which there is no counterpart in humans, e.g. accumulation of α_{2u}-globulin in the male rat kidney (Baetke *et al.* 1991).

The criteria for levels of concern for the short-term subchronic studies, assuming oral dosing, are intermediate between those for acute toxicity studies and 90-day subchronic toxicity studies (see below). To convert oral criteria into the corresponding criteria for dermal and inhalation routes of exposure see Appendix 1.

High Concern:

- Adaptive or nonspecific toxicity at doses < 20 mg/kg/day;

- Target organ or systemic toxicity at doses < 30 mg/kg/day.

Medium Concern:

- Adaptive or nonspecific toxicity at doses ≥ 20 but < 200 mg/kg/day;

- Target organ or systemic toxicity at doses ≥ 30 but < 300 mg/kg/day.

Low Concern:

- Adaptive or nonspecific toxicity at doses ≥ 200 mg/kg/day;

- Target organ or systemic toxicity at doses ≥ 300 mg/kg/day.

Endocrine Disruption Screening

Endocrine disruption screening does not have an established regulatory basis as of yet, but the Food Quality Protection and Safe Drinking Water Acts of 1996 have moved the EPA in that direction through its Endocrine Disruptor Screening Program. An endocrine disruption *in vitro* screening battery should consist of two assays for an initial assessment of estrogenic activity, i.e. estrogen receptor binding assay and transcriptional activation assay. These studies cost approximately $20,000, require approximately four months from initiation to final report, and utilize no animals. Normally, estrogenic activity would be evaluated first because experience shows that this aspect of the endocrine system is most commonly affected (Daston *et al.* 1997). However, if there are any reasons to suspect that the androgen or thyroid systems are susceptible, they should be screened as well as or instead of the estrogen system. Other *in vivo* screening assays are currently under development or need validation (see URL: http://www.epa.gov/; Federal Register: December 28, 1998, Volume 63, No. 248). Screening results demonstrating adverse endocrine effects may be predictive for reproductive, neurologic, immunologic or carcinogenic activity in long-term *in vivo* studies. At the present time, it is not possible to assign level of concern to the outcomes of the *in vitro* screening test for endocrine disrupters. As these tests are validated, the classification criteria will be developed.

Evaluation of Level 2 Testing and Determination of Data Needs

Upon completion of the Level 2 testing activities, a much more realistic picture of the potential toxicity of the new chemical should be available. Information on the acute toxicity of the chemical, its potential for eye and skin irritation and skin sensitization, its mutagenic potential, limited subchronic toxicity and some indications of potential for endocrine disruption will be available for review. At this time an external panel of experts (peer review panel) should be appointed to act as independent reviewers of the hazards posed by the chemical. The peer review panel membership expertise should encompass the areas of chemistry, exposure assessment and toxicology, in particular, as related to the new chemical. This peer review committee will be responsible for the ultimate recommendations for the safe use of the chemical by the Air Force. Historically, this function has been provided by the Committee on Toxicology of the National Academy of Sciences.

If the peer review committee finds that the concern level is high for any of the Level 2 study findings, then the use of the chemical by the Air Force should be reevaluated. High levels of concern do not rule out the potential use of a chemical; however, the consequences of these findings must be evaluated and requirements for projected chemical handling, levels of personal protective equipment required, and overall exposure criteria associated with industrial hygiene engineering concerns, will apply to the potential restrictions on use of the chemical considered. If further development of the chemical is warranted, then additional toxicological data will be required to fully appreciate the toxicological impact.

It should be noted that while primary irritation testing results are considered in the overall rating of a chemical, they should not dictate the overall rating of a chemical because industrial hygiene practices are available to mitigate toxicological hazards. In addition, positive mutagenicity results are not considered when selecting the overall level of concern for a chemical if upon further testing the chemical does not produce an oncogenic effect in an appropriate chronic animal study (see below).

Consensus opinion on Level 3 data needs should be determined based on the deliberations and decisions of the peer review committee. Advice of the panel should address specific data needs and any special focusing on particular endpoints identified in the Level 2 testing.

LEVEL 3 - INTERMEDIATE TOXICOLOGY STUDIES

Level 3 toxicology studies provide the second or intermediate tier of experimental toxicity data to identify potential human health hazards for the chemical of interest. The amount of data that need to be developed depends on the strategy developed as a result of Level 2 testing results. Generally, Level 3 testing is conducted to obtain more detailed information on hazards identified in Level 2 and to obtain information on toxicokinetics and developmental toxicity potential. The reader is referred to the EPA Health Effects Test Guidelines (U.S. EPA 1996) and OECD Guidelines for Testing of Chemicals (1998) for discussion of the specific protocol requirements of Level 3 studies.

Metabolism and Toxicokinetics

Metabolism and toxicokinetic studies are useful for determining the uptake of a chemical by the body from various exposure routes, distribution of the chemical in the organs and tissues of the body, and elimination of the chemical and its metabolites from the body. Also, metabolism and toxicokinetic studies are useful for setting dose levels for subchronic and chronic studies and for extrapolating toxicology data from animals to humans.

A toxicokinetic study typically costs $50,000 to $200,000, requires approximately four to twelve months from initiation to final report, and may utilize greater than 100 animals, depending on study design. Analytical methods are an important component of toxicokinetic studies. Although this report does not recommend specific levels of concern for results from toxicokinetic studies, issues such as a long half-life for a chemical or metabolism to a reactive chemical intermediate (e.g. epoxides, free radical, etc.) tend to raise the level of concern for a chemical. The purpose and principle of test method are described in OECD Guideline 417 "Toxicokinetics" (edited excerpts follow).

Introduction, Purpose, Scope, Relevance, Application, and Limits of Test.
Toxicokinetics embraces a number of investigative areas, which can be examined in various combinations and in various levels of depth.
Information from toxicokinetic studies on the absorption, distribution, excretion and metabolism of a test substance is desirable to aid in the evaluation and interpretation of toxicological data. Flexibility, taking into consideration the characteristics of the substance being investigated, is needed in the design of toxicokinetic studies. The actual study is designed to suit the particular substance in question.

Toxicokinetic studies may provide data useful for selecting appropriate dose levels for use in other toxicology studies.

Toxicokinetic studies can provide information on the amount and rate of absorption of the test substance, the pattern of distribution of the test substance among tissues, organs and fluid compartments, the reversible binding of the test substance to tissue sites and plasma proteins, the pattern and the rates of metabolism, the rates of excretion, and biochemical parameters (such as irreversible binding of the chemical with tissue or macromolecules, effects on metabolizing enzyme systems or depletion of endogenous non-protein sulfhydryl compounds, e.g. glutathione). It is not envisaged that all the aspects mentioned above will need to be investigated in every case.

Principle of the test method.
The test substance is administered by an appropriate route. Depending on the purpose of the study, the substance may be administered in single or repeated doses for defined periods to one or several groups of experimental animals. Subsequently, depending on the type of study, the substance and/or metabolites are determined by quantitative analytical chemical techniques in body fluids, tissues and/or excreta.

Genetic Toxicity

Additional genetic toxicity testing may be warranted if some of the results from *in vitro* studies conducted in Level 2 are equivocal or positive. The most commonly performed study under these circumstances is the *in vivo* mouse micronucleus test. The *in vivo* mouse micronucleus test can be conducted using a single or multiple dosing regimen. If the study is carried out at either a limit dose or the maximum tolerated dose, or if it can be demonstrated that the test substance is reaching the target tissue, the results of this study normally would override the results of *in vitro* assays for the purpose of risk characterization.

In vivo mammalian micronucleus

The *in vivo* mammalian micronucleus test typically costs $20,000, requires approximately three months from initiation to final report, and utilizes between 20 and 40 rats or mice. The purpose, initial considerations and principle of the test method are described in OPPTS 870.5395 "Mammalian Erythrocyte Micronucleus Test" (edited excerpts follow).

> **Purpose.** The mammalian *in vivo* micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents. The purpose of the micronucleus test is to identify substances that cause cytogenetic damage, which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.

> When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise anucleated erythrocyte cytoplasm. Visualization of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage.

> **Initial considerations.** The bone marrow of rodents is routinely used in this test since polychromatic erythrocytes are produced in that tissue. The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations. Micronuclei can be distinguished by a number of criteria. These include identification of the presence or absence of a kinetochore or centromeric DNA in the micronuclei. The frequency of micronucleated immature (polychromatic) erythrocytes is the principal endpoint. The number of mature (normochromatic) erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of the assay when animals are treated continuously for 4 weeks or more. This mammalian *in vivo* micronucleus test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and

DNA-repair processes although these may vary among species, among tissues and among genetic endpoints. An *in vivo* assay is also useful for further investigation of a mutagenic effect detected by an *in vitro* system.

If there is evidence that the test substance, due to removal from blood by a hepatic first pass effect or a reactive metabolite produced in an organ will not reach the target tissue, it is not appropriate to use this test.

Principle of the test method. Animals are exposed to the test substance by an appropriate route. If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and preparations made and stained. When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained. For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analyzed for the presence of micronuclei.

The level of concern for genotoxicity is assigned as described below based on the additional evidence provided by the *in vivo* testing results.

High Concern: Positive in *in vivo* tests.

Medium Concern: Several positive assay results but overall conflicting findings across
                    assays in test battery.

Low Concern: Negative in all *in vitro* and *in vivo* tests and QSAR analysis.

Subchronic Toxicity

A ninety-day subchronic toxicity study is conducted following a 28-day repeated dose range-finding study by the appropriate route. Subchronic toxicity studies are performed to characterize the human health hazard from long term repeated exposure to chemicals. These studies include daily clinical observations, periodic biochemical evaluations of serum and urine, blood cell measurements, observation of gross pathology at necropsy and histopathology, and measurements of body weights and organ weights. Subchronic studies are designed to identify the NOAEL for the chemical, target organs, the pathological nature of the adverse effects and dose-response relationships for the effects. Data from subchronic toxicity studies, when extrapolated to humans, provide a basis to establish acceptable occupational exposure limits.

The subchronic toxicity study can be used to evaluate neurotoxicity through observations of behavior and nervous system histopathology, immunotoxicity through evaluation of white blood cells and immune system histopathology and reproductive toxicity through evaluation of reproductive organs by gross observations or histopathology. However, the study design of the basic subchronic toxicity study can be modified to include special evaluations to examine more thoroughly the effects of a chemical on these special endpoints. EPA testing guidelines are available for the detailed functional and histopathological assessment of neurotoxicity or immunotoxicity during the course of subchronic toxicity testing. Also, OECD testing guidelines

provide a screening protocol for the simultaneous evaluation of systemic toxicity, reproductive toxicity and developmental toxicity potential through a detailed functional assessment. Whereas the EPA neurotoxicity and immunotoxicity tests are considered to be definitive, the OECD combined repeated dose toxicity study with the reproductive/developmental toxicity screening test is considered to be a preliminary assessment.

An oral 90-day subchronic toxicity study typically costs $125,000, requires approximately nine months from initiation to final report, and utilizes between 80 and 160 rats. An inhalation 90-day subchronic toxicity study typically costs $200,000, requires approximately ten months from initiation to final report, and utilizes 80 to 160 rats. A dermal 90-day subchronic toxicity study typically costs $130,000, requires approximately nine months from initiation to final report, and utilizes between 80 and 160 rats. Neurotoxicity or immunotoxicity evaluations may be added to the subchronic toxicity study design at an additional cost of about $75,000 or $35,000 respectively.

Effects observed in 90 day subchronic toxicity studies should be grouped using the categories of adaptive/non specific effects, target organ/systemic effects or not relevant effects (as described in the section on short term subchronic tests above) to establish the level of concern. In general, the dose levels that trigger a specific level of concern for an adaptive or nonspecific effect versus a target organ or systemic effect will be two times lower in subchronic studies.

The levels of concern for the 90-day subchronic toxicity studies are (see Appendix 1 to convert oral dosages into equivalent dosages for inhalation and dermal routes of exposure):

High Concern:

- Adaptive or nonspecific toxicity at doses < 10 mg/kg/day;

- Target organ or systemic toxicity at doses < 20 mg/kg/day.

Medium Concern:

- Adaptive or nonspecific toxicity at doses ≥ 10 but < 100 mg/kg/day;

- Target organ or systemic toxicity at doses ≥ 20 but < 200 mg/kg/day.

Low Concern:

- Adaptive or nonspecific toxicity at doses ≥ 100 mg/kg/day;

- Target organ or systemic toxicity at dose ≥ 200 mg/kg/day.

Developmental Toxicity

Developmental toxicity is any adverse effect observed in the fetus/neonate induced during the period from conception through puberty. The major types of developmental toxicity are embryo-lethality, structural abnormalities, altered growth and functional deficiencies. Chemical effects on the developing fetus may be mediated through toxicity in the parents. These effects are not generally considered to be developmental toxicity. However, effects in the fetus that result from

the direct interaction of the chemical with developmental processes are attributed to developmental toxicity and generally raise the level of concern.

A developmental toxicity study typically costs $100,000, requires approximately eight months from initiation to final report, and utilizes between 100 and 150 rats. The purpose and principle of the test method are described in OPPTS 870.3700 "Prenatal Developmental Toxicity Study" (edited excerpts follow).

Purpose. This guideline for developmental toxicity testing is designed to provide general information concerning the effects of exposure of the pregnant test animal on the developing organism; this may include death, structural abnormalities, or altered growth and an assessment of maternal effects.

Principle of the test method. The test substance is administered to pregnant animals at least from implantation to one day prior to the expected day of parturition. Shortly before the expected date of delivery, the pregnant females are terminated, the uterine contents are examined, and the fetuses are processed for visceral and skeletal evaluations.

For developmental toxicity, the reported effects are grouped into two general categories depending on whether they represent fetotoxicity or developmental toxicity, and the level of concern is assigned based on the dose level at which these effects occur. Listed below are the guidelines for establishing the level of concern for developmental effects.

High Concern:

- Fetotoxicity at doses < 10 mg/kg/day;

- Malformations or variations at doses < 100 mg/kg/day.

Medium Concern:

- Fetotoxicity at doses ≥ 10 but < 100 mg/kg/day;

- Malformations or variations at doses ≥ 100 but < 1000 mg/kg/day.

Low Concern:

- Fetotoxicity at doses ≥ 100 mg/kg/day;

- Malformations or variations at doses ≥ 1000 mg/kg/day.

Levels of concern that are established based on developmental toxicity or fetotoxicity generally will decrease one level if the effects observed are accompanied by maternal toxicity.

Endocrine Disruption

As discussed above, endocrine disruption screening does not have, as yet, an established regulatory basis. The proposed endocrine disruption *in vivo* screening battery consists of one assay for an initial assessment of estrogenic activity, i.e. rodent 3-day uterotrophic assay. This study costs approximately $10,000, requires approximately four months from initiation to final report, and utilizes 10 to 40 animals. If there are any reasons to suspect that the androgen or thyroid systems are susceptible, they should be screened as well as or instead of the estrogen system. Other *in vivo* screening assays are currently under development or need validation (see URL: http://www.epa.gov/; **Federal Register**: December 28, 1998, Volume 63, No. 248).

Evaluation of Level 3 Testing and Determination of Data Needs

At the completion of Level 3 testing a rather complete understanding of the toxicity of the chemical under consideration is available. The subchronic and developmental toxicity studies provide a significant database that can be interpreted by the peer review committee to predict the potential toxicity of the chemical under expected conditions of use. In most cases, the toxicity data available at this juncture are more than adequate for toxicologists to evaluate safe levels of exposure and for risk managers to define necessary industrial hygiene practices that should be employed by Air Force personnel. Level 4 data needs should only be required for chemicals that are expected to be widely used by the Air Force, that will be used in large volumes or that have a high probability of resulting in general exposure of the public. If mutagenicity studies are equivocal and significant exposure of unprotected personnel is possible, then lifetime studies in two species may be required. At this stage, the added value of the Level 4 test is not warranted unless there are outstanding concerns in the minds of the toxicologists resulting from the previous studies.

LEVEL 4 – ADVANCED TOXICOLOGY STUDIES

The reader is referred to the EPA Health Effects Test Guidelines (U.S. EPA 1996) and OECD Guidelines for Testing of Chemicals (1998) for discussion of the specific protocol requirements of Level 4 studies.

Chronic Toxicity/Carcinogenicity (Oncogenicity)

Chronic toxicity/carcinogenicity studies are not generally conducted for nonregulated chemicals for which the exposures are primarily occupational. For most chemicals, the subchronic toxicity study(ies) and an *in vitro* and *in vivo* genetic toxicity test battery are sufficient to assess the potential for long-term toxicity from repeated exposures and oncogenicity. If chronic toxicity/carcinogenicity studies are conducted, it is important to keep in mind the extreme conditions (i.e. lifetime exposure at the maximum tolerated dose) used to elicit a tumorigenic response when interpreting the results.

A chronic toxicity/carcinogenicity study typically costs $900,000, requires approximately 36 months from initiation to final report, and utilizes a minimum of 480 rats or mice. The purpose of

this study type is described in OPPTS 870.4300 "Combined Chronic Toxicity/Carcinogenicity" (edited excerpts follow).

> **Purpose.** The objective of a combined chronic toxicity/carcinogenicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. The application of this guideline should generate data that identify the majority of chronic and oncogenicity effects and determines dose-response relationships. The design and conduct should allow for the detection of neoplastic effects and a determination of the carcinogenic potential as well as general toxicity, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

Chronic Toxicity

As in the subchronic toxicity studies, the results of the chronic toxicity studies are classified into the three categories – adaptive/non-specific effects, target organ/systemic effects and nonrelevant effects – prior to assigning a level of concern. In general, the dose levels that establish the levels of concern for an adaptive or nonspecific effect versus a target organ or systemic effect will be ten times lower for chronic studies. The levels of concern for chronic toxicity testing are:

High Concern:

- Adaptive or nonspecific toxicity at doses < 1 mg/kg/day;

- Target organ or systemic toxicity at doses < 10 mg/kg/day.

Medium Concern:

- Adaptive or nonspecific toxicity at doses ≥ 1 but < 10 mg/kg/day;

- Target organ or systemic toxicity at doses ≥ 10 but < 100 mg/kg/day.

Low Concern:

- Adaptive or nonspecific toxicity at doses ≥ 10 mg/kg/day;

- Target organ or systemic toxicity at doses ≥ 100 mg/kg/day.

Carcinogenicity (or Oncogenicity)

For oncogenicity, the reported effects will be grouped into two general categories depending on the likelihood for oncogenic effects to be mediated through genotoxic or nongenotoxic mechanisms. A high level of concern is assigned if there is evidence of genotoxic oncogenicity, regardless of the dose. The level of concern for nongenotoxic oncogenicity is based on the dose level at which the effect is observed. Listed below are the guidelines for establishing a level of concern for oncogenicity.

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High Concern:

- Evidence for genotoxic oncogenicity regardless of the dose;

- Evidence of nongenotoxic oncogenicity at doses < 100 mg/kg/day.

Medium Concern:

- Evidence of nongenotoxic oncogenicity at doses ≥ 100 but < 1000 mg/kg/day.

Low Concern:

- Evidence of nongenotoxic oncogenicity at doses ≥ 1000 mg/kg/day.

The determination of whether or not the oncogenic effect is mediated by genotoxic or nongenotoxic mechanisms is determined on the basis of other effects in the target organ and the results of the mutagenicity tests. Consideration is also given to the relevance of the oncogenic effect to human health when selecting the level of concern since some nongenotoxic oncogenic effects observed in animals studies are not expected to occur in humans, e.g. kidney tumors produced through the accumulation of α_{2u}-globulin in rats.

Reproductive Toxicity

Reproductive toxicity covers all phases of the reproductive cycle, and includes impairment of male or female reproductive function or capacity and the induction of nonheritable adverse effects on offspring (including death, growth retardation, structural abnormalities and functional effects). The two-generation reproductive toxicity study design goes beyond the type of reproductive assessment discussed in the Level 3 toxicology studies section, in that it is particularly sensitive to elucidating endocrine disruption and transgenerational effects.

Reproduction and fertility studies typically cost $450,000 (2-generation study) or $150,000 (fertility study), require approximately twelve or six months, respectively, from initiation to final report, and utilize about 150 or 200 rats each. The purpose and principle of the test method are described in OPPTS 870.3800 "Reproduction and Fertility Effects" (edited excerpts follow).

Purpose. This guideline for two-generation reproductive toxicity testing is designed to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, target organs in the offspring, and preliminary data on prenatal and postnatal developmental toxicity and serve as a guide for subsequent tests. Additionally, since the study design includes *in utero* as well as postnatal exposure, this study provides the opportunity to examine the susceptibility of the immature/neonatal animal. For further information on functional deficiencies and developmental effects, additional study segments can be

incorporated into the protocol, utilizing the guidelines for developmental toxicity or developmental neurotoxicity.

Principle of the test method. The test substance is administered to parental animals prior to and during their mating, during the resultant pregnancies, and through the weaning of their F_1 offspring. The substance is then administered to selected F_1 offspring during their growth into adulthood, mating, and production of an F_2 generation, until the F_2 generation is weaned.

For reproductive toxicity, the level of concern is assigned as described below depending on the dose level at which significant reproductive effects are observed. In this case, the dose levels used to define the levels of concern are the same as those used for chronic target organ or systemic effects.

High Concern: Evidence of reproductive toxicity at doses < 10 mg/kg/day.

Medium Concern: Evidence of reproductive toxicity at doses ≥ 10 but < 100 mg/kg/day.

Low Concern: Evidence of reproductive toxicity at doses ≥ 100 mg/kg/day.

Levels of concern that are established based on reproductive effects likely will decrease one level if the effects observed are accompanied by parental toxicity.

Developmental Neurotoxicity

Developmental neurotoxicity examines the potential functional and morphological effects to the nervous system that may occur in the offspring from maternal exposure to a chemical during pregnancy and lactation. The study is unique in comparison to the developmental toxicity and neurotoxicity study designs discussed in the potential Level 3 toxicology studies section because 1) special evaluations of neurotoxicity are performed that are not performed in the developmental toxicity study, and 2) offspring are evaluated, whereas young adults are evaluated in the neurotoxicity study design. The levels of concern criteria described for subchronic toxicity (Level 3) and/or developmental toxicity (Level 3) are appropriate to use for evaluation of developmental neurotoxicity.

A developmental neurotoxicity study typically costs $150,000, requires approximately nine months from initiation to final report, and utilizes between 100 and 150 rats. The purpose and principle of the test method are described in OPPTS 870.6300 "Developmental Neurotoxicity Study (edited excerpts follow).

> **Purpose.** In the assessment and evaluation of the toxic characteristics of a chemical substance or mixture (test substance), determination of the potential for developmental neurotoxicity is important. This study is designed to develop data on the potential

functional and morphological hazards to the nervous system that may arise in the offspring from exposure of the mother during pregnancy and lactation.

Principle of the test method. The test substance is administered to several groups of pregnant animals during gestation and early lactation, one dose level being used per group. Offspring are randomly selected from within litters for neurotoxicity evaluation. The evaluation includes observations to detect gross neurologic and behavioral abnormalities, determination of motor activity, response to auditory startle, assessment of learning, neuropathological evaluation, and brain weights. This protocol may be used as a separate study, as a follow-up to a standard developmental toxicity and/or adult neurotoxicity study, or as part of a two-generation reproduction study, with assessment of the offspring conducted on the second (F_2) generation.

Evaluation of Level 4 Testing and Development of Data Needs

Level 4 toxicology studies are considered the definitive animal studies for chronic and/or reproductive toxicity. Upon completion of Level 4 studies a relatively complete toxicology dossier for the chemical of concern is available. The database should be adequate for conducting a standard risk assessment, which would allow for the establishment of recommended safe levels of exposure (i.e. exposure standards). The role of the peer review committee at this stage is to determine if the database is adequate for such an activity to proceed. If adequate data are available, then the committee should proceed on to the risk assessment. If essential data are still needed, the peer review committee should identify the data requirements and the additional research implemented.

RISK ASSESSMENT

When adequate toxicology data are available, it is preferred to use risk assessment rather than hazard-based assignment of levels of toxicological concern for decision-making related to chemical safety and/or the need for personal protection from occupational chemical exposures. Whereas hazard-based assignment of levels of toxicological concern may be used early in the development or use of a new chemical, as a chemical and its use patterns become established, risk assessment is the more appropriate methodology to employ.

The risk assessment procedure described in this report, i.e. comparison of a virtually safe dose (VSD) to an estimated human dose (EHD), is the procedure most commonly applied by regulatory authorities and the scientific community at large (Lewis *et al.* 1990). Often, other terminology is used, e.g. acceptable daily intake (ADI) and estimated daily intake (EDI), but the process is the same as that described below.

Ideally, VSDs and EHDs are derived from experimental data. The key elements of a noncancer risk assessment are as follows:

1. Health hazard characterization: Health hazard characterization is performed. Health hazard characterization begins with the identification of the most biologically relevant health effects

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associated with exposure to a chemical in laboratory animal toxicology studies, together with information on doses likely to elicit these effects. Health hazard characterization requires gathering and evaluating information obtained relating to hazard identification and dose-response assessment (in most cases the data developed in Level 2 and 3 studies). Hazard identification means identifying the potentially dangerous properties of a chemical. Dose-response assessment is the characterization of the relationship between the dose (exposure) to a chemical and the anticipated incidence of an adverse health effect in an exposed population.

2. <u>NOAEL determined for critical health effect</u>: From health hazard characterization, a no-observed-adverse-effect-level (NOAEL) is determined for the critical health effect. A NOAEL is the dose of a chemical at which there are no statistically or biologically significant increases in the frequency or severity of adverse effects between the exposed laboratory animal population and its appropriate control group. A no-observed-effect-level (NOEL) is subtly different from a NOAEL in that a NOEL is determined by any effect in the exposed laboratory animal population as compared to its appropriate control group, even if that effect is not considered to be adverse. The critical health effect refers to the specific adverse health effect on which the risk characterization is based. That critical health effect may not be the most sensitive of all effects, but it is the most sensitive adverse effect (i.e. of toxicological significance). The U.S. EPA defined adverse effect as functional impairment or pathological lesion that may affect the performance of the organism as a whole or which reduces the ability of the organism to respond to an additional challenge (cited in: Dourson and Stara 1983).

3. <u>Derivation of VSD</u>: The NOAEL from a toxicology study is then adjusted by uncertainty factors to derive VSD for humans. Applying uncertainty factors to the highest NOAEL from appropriate toxicology studies generally derives the VSD for humans. The uncertainty factor should take into consideration the following:

- Known differences between laboratory animals and humans, and the uncertainties of extrapolating animal data to humans;
- Variations in the sensitivity of the exposed human population;
- Strength of evidence that a chemical presents a real hazard to human health;
- Type and severity of the putative adverse health effect;
- Potency of the toxic agent; and
- Quality of the toxicology database, including known differences between experimental conditions and real life exposures.

4. <u>Derivation of EHD</u>: Exposure assessment is performed. The objective of exposure assessment is to determine an EHD, which is the predicted average amount of a chemical that an individual in a human population will receive as the result of an activity that places them in contact with a chemical. The estimate is based on identifying and quantifying the potential exposures in terms of magnitude, frequency, duration and route of exposure for a chemical to humans.

5. <u>Risk characterization</u>: Risk characterization is performed. Risk characterization is a description of the nature and magnitude of health risk. The description combines results of

hazard characterization and exposure assessment, and describes the uncertainty associated with each step. Mathematically this comparison can be expressed as:

$$\text{Relative Risk} = \frac{\text{EHD}}{\text{VSD}}$$

- In cases where the relative risk ratio is much greater than "1", risk is high because the EHD is higher than the VSD.
- In cases where the relative risk ratio is much less than "1", risk is low because the EHD is lower than the VSD.
- In cases where the relative risk ratio is about "1", risk is indeterminate and improved toxicology and/or exposure data are probably needed and/or engineering or personnel protection requirements must be established to reduce the numerator until the ratio is acceptable.

Results of the risk assessment provide critical information to the risk manager. For instance, results of the risk assessment may help to establish the need for occupational monitoring and surveillance, establish the need for protective equipment in the workplace, and identify potential concerns for transport, storage and disposal. Of course, in many cases, the results of the risk assessment will serve to demonstrate the lack of health hazards for the new chemical, alleviating concerns for occupational exposure.

Risk assessment is an iterative process whereby the availability of new toxicology and/or exposure data can be used to improve the quality and accuracy of the risk characterization. Therefore, the process described in this section is applicable to all stages in the evaluation of a new chemical as new toxicology and exposure data are developed.

COST AND TIMING ESTIMATES FOR TOXICOLOGY TESTING

The full evaluation of the toxicity of a chemical is a costly and time consuming process. Table 4 provides a summary of the cost and duration of the proposed tests discussed above. Since the level of confidence in our knowledge about the toxicity of a chemical is related to the extent of the testing and evaluation conducted, it is clear that significant resources must be committed to this activity in order to attain a minimal level of knowledge to make rational decisions concerning the deployment of new materials.

Toxicology Study Type	Cost Estimates ($)	Timing Estimates (months)
Acute toxicity battery		
• Acute oral toxicity	5,000	3
• Acute dermal toxicity	5,000	3
• Acute inhalation toxicity	20,000	3
• Skin irritation	1,000	3
• Eye irritation	1,000	3
• Skin sensitization	10,000	3
Genetic toxicity battery		
• *In vitro* bacterial gene mutation	5,000	3
• *In vitro* mammalian cell cytogenetics	20,000	3
• *In vitro* mammalian cell gene mutation	20,000	3
• *In vivo* mammalian micronucleus	20,000	3
Repeated dose toxicity		
• Oral		
– 14- or 28-Day range-finding	35,000 – 60,000	3 – 4
– 90-Day subchronic toxicity	125,000	9
• Dermal		
– 21- or 28-Day range-finding	45,000 – 60,000	3 – 4
– 90-Day subchronic toxicity	130,000	9
• Inhalation		
– 14- or 28-Day range-finding	70,000 – 90,000	4 – 5
– 90-Day subchronic toxicity	200,000	10
• Neurotoxicity[1]	75,000	10
• Immunotoxicity[1]	35,000	10
• Reproduction[2]	70,000	6
Endocrine disruption screening battery		
• *In vitro* screening	20,000	4
• *In vivo* screening	10,000	4
Metabolism and pharmacokinetics (toxicokinetics)	50,000 – 200,000	4 – 12
Developmental toxicity	100,000	8

Table 4: Summary of Cost and Timing Estimates Summary for Data Development Plan

Toxicology Study Type	Cost Estimates ($)	Timing Estimates (months)
Developmental toxicity	100,000	8
Developmental neurotoxicity	150,000	9
Reproduction and fertility	150,000 – 450,000	6 – 12
Chronic toxicity and oncogenicity	900,000	36
Mechanistic research	TBD	TBD

Table 4: Summary of Cost and Timing Estimates Summary for Data Development Plan (continued).
[1]Conducted as part of a 90-day subchronic toxicity study. [2]Conducted using the OECD combined repeated doses toxicity study with the reproductive/developmental toxicity screening test.
TBD – To be determined when the research program is designed.
Note: Cost estimates are average values and timing estimates assume optimal circumstances; actual cost and timing are determined by the final protocol and the laboratory used for the study.

	Time (Months)											
Level 1	1	2	3	4	5	6	7	8	9	10	11	12
Database Searching	▓	▓	▓									
QSAR Analysis	▓											
In Vitro Cytotoxicity	▓	▓	▓									
In Vitro Mutagenicity (Bacterial)	▓	▓	▓									
Level 1 Evaluation				▓								
Level 2	1	2	3	4	5	6	7	8	9	10	11	12
Acute Toxicity					▓	▓	▓					
Skin Irritation					▓	▓	▓					
Eye Irritation					▓	▓	▓					
Skin Irritation					▓	▓	▓					
28-Day Range finding Study (One route of exposure)					▓	▓	▓	▓				
In Vitro Mutagenicity (Mammalian cells)					▓	▓	▓					
In Vitro Cytogenicity (Mammalian cells)					▓	▓	▓					
In Vitro Endocrine Disruption Screen					▓	▓	▓	▓				
Level 2 Evaluation									▓			

Figure 2. Gant chart describing optimal schedule for toxicity testing.

	Time (Months)											
Level 3	10	11	12	13	14	...	17	18	19	20	21	22
Toxicokinetics	▓	▓	▓	▓	▓	▓	▓					
90-Day Subchronic Study (One route of exposure)	▓	▓	▓	▓	▓	▓	▓	▓	▓			
Neurotoxicity Screen	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓		
Immunotoxicity Screen	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓		
Reproductive Screen	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓		
In Vivo Mammalian Micronucleus	▓	▓	▓	▓								
In Vivo Endocrine Disruption Screen	▓	▓	▓	▓								
Developmental Toxicity	▓	▓	▓	▓	▓	▓	▓	▓				
Level 3 Evaluation												▓
Level 4	25	...	...	30	31	32	33	34	35	...	...	48
Chronic Toxicity/Carcinogenicity	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓
Reproductive Toxicity	▓	▓	▓	▓	▓	▓	▓	▓				
Developmental Neurotoxicity	▓	▓	▓	▓								

Figure 2. Gant chart describing optimal schedule for toxicity testing (continued).

Figure 2 is a Gant chart for the toxicity testing process. This chart further emphasizes the lead-time needed to attain the maximum level of confidence in our understanding of the toxicity of a new chemical. In general, if funds are available, Level 1 evaluations can be completed in about 6 months. Completion of all testing through Level 2 can be accomplished in about 1 year. Completing Level 3 testing will take about 2 years. If however, Level 4 chronic studies are required and then the entire testing program could take up to 4 years. Obviously, if the chronic studies are started earlier, the schedule will be compressed. The gist of all of this is that adequate toxicity testing will require several years and significant financial resources. However, short-circuiting the process to save time and money leaves you vulnerable to extensive residual costs for unforeseen health effects and remediation.

CONCLUSIONS AND RECOMMENDATIONS

1) Under no circumstances is there 100% certainty that a chemical is safe. The proposed testing strategy is designed to maximize the level of confidence in understanding the toxicology of chemicals of interest to the Air Force. The higher the level of testing conducted, the greater becomes the confidence that no unexpected health effects will arise when the chemical is used.

2) Information for hazard characterization and risk assessment should be developed logically and sequentially, beginning with basic information, and then moving on to more detailed and

complex information. Iterative evaluation of data is necessary to guide the data development process for health hazard characterization.

3) Chemicals with toxicological classification of high concern for any endpoint require immediate action, those chemicals with toxicological classifications of low concern for all endpoints require minimal action, and chemicals with toxicological classifications of medium concern require case-by-case assessment using expert professional judgement to determine what additional actions are required.

4) A small internal chemical steering committee should be formed to track and manage the evaluation process for new chemicals of interest to the Air Force. Under the direction of the chemical steering committee, the Level 1 activities should be conducted and the information collected should be entered into a toxicity database for easy reference.

5) A theoretical analysis of the occupational exposure potential for the new chemical should be undertaken at Level 1 by industrial hygienists. If the new chemical eventually will be manufactured and/or handled in relatively large volumes, or if the health hazard evaluation identifies areas of concern, a more detailed occupational exposure assessment may be warranted when the chemical is deployed to the field.

6) In many cases, structure-activity analysis can not be performed without a high degree of uncertainty, and insufficient human health hazard or animal toxicology data are available on close structural analogs of the new chemical to conduct a preliminary health hazard characterization. In instances such as those, *in vitro* screening tests may be advisable to develop the data necessary for a preliminary health hazard characterization for the new chemical. Development of new QSAR and *in vitro* testing methods are needed and should be supported.

7) Literature searches should be conducted in three phases. In the first phase, the biographical databases are searched for all relevant toxicological citations on the identified analog chemicals. The next phase of the literature search is to collect and review the information in the toxicity databases. The final phase of the search strategy is to search specific databases and/or develop specific strategies to locate studies of a particular type to fulfill data gaps.

8) At the completion of the Level 1 activities, the chemical steering committee should provide specific guidance for the type of toxicological data needed to satisfy the requirements of all stakeholders. The proposed testing strategy, based on the multilevel tier approach proposed in this report should identify the most likely toxicological issues related to the particular chemical and its expected use. Taking into consideration the expected route of exposure and potential toxicological properties, a checklist of recommended toxicity tests should be prepared identifying tests and crosslisting tests with anticipated toxicological issues.

9) Upon completion of the Level 2 testing activities, an external panel of experts (peer review panel) should be appointed to act as independent reviewers of the hazards posed by the

chemical. The peer review panel will be responsible for the ultimate recommendations for the safe use of the chemical by the Air Force.

10) If the peer review committee finds that the concern level is high for any of the Level 2 study findings, then the use of the chemical by the Air Force should be reevaluated. High levels of concern do not rule out the potential use of a chemical; however, the consequences of these findings must be evaluated and the potential restrictions on use of the chemical considered. If further development of the chemical is warranted, then additional toxicological data will be required to fully appreciate the scope of potential toxicological impacts.

11) Hazard-based assignment of levels of toxicological concern should be used early in the development or use of a new chemical. When adequate toxicology data become available (following Level 2, 3 and/or 4 studies), it is recommended to use risk assessment rather than hazard-based assignment of levels of toxicological concern for decision-making related to chemical safety and/or the need for personal protection from occupational chemical exposures.

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APPENDIX 1:

Conversions of Repeated Dose Toxicity Studies Data for Route-to-Route Extrapolation

In order to categorize the data from repeated exposure toxicity studies, the nature of the effects and the doses at which they are observed are considered. The dose levels and exposure concentrations used in the longer-term toxicity studies are converted to mg/kg/day and adjusted, if appropriate, so that direct comparisons and extrapolations may be made between the results of studies that employ different routes of administration or exposure.

Oral to inhalation extrapolation

For inhalation study data, exposure concentrations are converted from mg/kg/day to mg/l or ppm using the following formula. This conversion assumes 100% absorption and bioavailability following inhalation exposure.

$$\text{Air Concentration (mg/l)} = \frac{\text{Dose (mg/kg/day)} * \text{Body Weight (kg)}}{\text{Minute Volume (l/min)} * \text{Minutes of Exposure per Day (min/day)}}$$

$$\text{Air Concentration (ppm)} = \frac{\text{Air Concentration (mg/l)} * 24,450}{\text{Molecular Weight}}$$

The approximate body weights and minute volumes for several species commonly used in inhalation studies are summarized below.

Species	Body Weight (kg)[1]	Minute Volume (l/min)
CD® rat	0.35	0.235
CD-1® mouse	0.03	0.039
Golden hamster	0.40	0.059
Guinea pig	0.45	0.085

[1]Assumes that animals are young adults, approximately 12 weeks of age.

The table below summarizes the unit conversions between oral dose levels in mg/kg/day and air concentrations in mg/l for some commonly used dose levels in rats, mice and hamsters.

Conversion of Oral Dose Levels
to Air Concentration Values

Dose (mg/kg/day)[1]	Corresponding Air Concentration (mg/l)		
	Rat	Mouse	Hamster
1	0.004	0.002	0.02
10	0.04	0.02	0.2
20	0.08	0.04	0.4
100	0.4	0.2	2
1000	4	2	20

[1]An exposure duration of six hours/day was assumed for this calculation.

Oral to dermal extrapolation

For dermal study data, a correction factor is employed to accommodate potential differences in bioavailability following dermal exposure compared to bioavailability following oral administration or inhalation exposure, both of which are assumed to be 100%. For example, if the level of Medium Concern is set at ≥ 20 but < 200 mg/kg/day for subchronic oral toxicity studies and 50% of the chemical is expected to penetrate the skin, the criteria level via the dermal route would be ≥ 40 but < 400 mg/kg/day.